Metal regulation of the E. faecalis efaCBA operon

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Metal Regulation of the *E. faecalis* *efaCBA* Operon

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Abstract

The EfaA protein of *Enterococcus faecalis* has been implicated as a virulence factor in enterococcal endocarditis. The enterococcal *efaCBA* operon encodes the components of an ABC-type transporter that is up-regulated in the presence of serum. This operon is homologous to the cluster 9 family of ABC transporters, and in particular to a sub-cluster whose members either are thought or have been demonstrated to be involved in manganese uptake. Some of these operons have been shown to be regulated by DtxR-like proteins in a metal-dependent manner. In this study, expression of the *efaCBA* operon, encoding a putative manganese transporter, is demonstrated to be regulated by the divalent transition metal cation Mn^{2+}. Under Mn^{2+}-limiting conditions, expression of EfaA and transcription of *efaCBA* were derepressed. Conversely, in the presence of ≥10 μM Mn^{2+}, expression of EfaA protein and transcription of *efaCBA* were repressed. Other divalent transition metal ions such as Fe^{2+} and Zn^{2+} had no effect. A Mn^{2+}-responsive transcriptional regulator of the *efa* operon was purified and characterised as a 222-amino-acid residue polypeptide, designated EfaR, which had 39% identity to the *S. gordonii* ScaR. Purified EfaR protein bound to the *efaC* promoter region *in vitro* in the presence of Mn^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+}. The protein binding sites were localised by DNA footprint analysis to two 14 bp motifs similar to the *S. epidermidis* SirR and the *C. diphtheriae* DtxR binding sequences adjacent to the −35 and −10 promoter signatures. Computer-based analysis of the unannotated *E. faecalis* V583 genome revealed the presence of putative EfaR binding sequences in a number of genes, suggesting that EfaR may have a global regulatory role. Taken together, the results identify a new DtxR-like Mn^{2+}-sensing regulator in *E. faecalis*, EfaR, which regulates the expression of EfaCBA, a virulence factor implicated in enterococcal endocarditis and likely Mn^{2+} permease.

Table of Contents

Abbreviations

ABC	ATP-binding cassette
APS	Ammonium persulphate
Amp	Ampicillin
AP	Alkaline Phosphatase
AS	Aggregation substance
ATP	Adenosine triphosphate
BHI	Brain heart infusion medium
BHY	Brain heart infusion and 1% yeast extract medium
BSA	Bovine serum albumin
Ca	Calcium
Cam	Chloramphenicol
Cd	Cadmium
CIAP	Calf intestinal alkaline phosphatase
Co	Cobalt
Cu	Copper
Da	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DUTP	Deoxyuridine triphosphate
ECM	Extracellular matrix proteins
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EMSA	Electrophoretic mobility shift assay
EPB	Electroporation buffer
FCS	Foetal calf serum
Fe	Iron
G6PD	Glucose-6-phosphate 1-dehydrogenase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMP	Hydrophobic transmembrane protein
IE	Infective endocarditis
IPTG	Isopropyl β-D-thiogalactopyranoside
IR	Inverted repeat
IV	Intravenous
Kan	Kanamycin

LB	Luria Bertani broth
M	Mole(s) per litre
MIC	Minimum inhibitory concentration
Mg	Magnesium
mg	Milligram
ml	Millilitre
Mn	Manganese
MOPS	3-[N-morpholino]-2-hydroxypropanesulphonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MWCO	Molecular weight cut-off
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
ng	Nanogram
Ni	Nickel
NRAMP	Natural resistance-associated macrophage protein
NTP	Nucleoside triphosphate
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidenedifluouride
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGM17	M17 medium supplemented with 0.5 M sucrose and 5% glycine
SOD	Superoxide dismutase
SSC	3 M sodium chloride, 0.3 M sodium citrate, pH 7.0
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	0.9% (w/v) NaCl, 10 mM Tris, pH 7.4
TE	Tris·Cl-EDTA buffer
TEMED	N,N,N,'N'-Tetramethylethylene diamine
Tet	Tetracycline
TIGR	The Institute for Genomic Research
Tris	Tris(hydroxymethyl) aminomethane
U	Unit
UTI	Urinary tract infection
TYHG	Tryptone yeast extract HEPES-buffered glucose medium
V	Volt(s)
v/v	Volume by volume
w/v	Weight by volume
X-Gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside)
YE	Yeast extract
Zn	Zinc

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Chapter 1: Introduction

The Enterococci

Enterococci are able to grow and survive in a wide range of environmental conditions, and so have been found in a variety of ecological niches ranging from soil, food, water and plants to the gastrointestinal tracts of mammals, birds and reptiles. In humans, the enterococci are typically found living as commensals in the gut and faeces, the lower gastrointestinal tract being the main habitat. They have also been isolated from the oral cavity, gall bladder, urethra and vagina (Morrison *et al.*, 1997).

The enterococci are Gram-positive cocci, occurring either singly, in pairs, or in short chains. They are non-spore-forming, facultatively anaerobic, usually catalase-negative (a pseudocatalase is sometimes produced, resulting in a weak effervescence) and the G+C content of their DNA ranges from 37 to 45 mol%. Most strains are able to grow at temperatures ranging from 10°C to 45°C but optimal growth occurs at 35°C. The enterococci were once regarded as belonging to the genus *Streptococcus* (where they were classed as group D streptococci), but genetic evidence from DNA-DNA, DNA-rRNA hybridisations and 16S rRNA sequencing demonstrates that *Streptococcus faecalis* and *Streptococcus faecium* were sufficiently distinct from other streptococci to warrant a separate genus (Schleifer and Kilpper-Balz, 1984). Since then, 15 other species have been proposed for inclusion in the genus (Hardie and Whiley, 1997). The sixteen species currently

accepted to belong to genus *Enterococcus* are: *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus dispar*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus gallinarum*, *Enterococcus malodoratus*, *Enterococcus mundtii*, *Enterococcus pseudoavium*, *Enterococcus raffinosus*, *Enterococcus saccharolyticus*, and *Enterococcus sulfureus* (Morrison *et al.*, 1997). Analysis of 16S rDNA suggests that most of these species can be divided among four species groups (Williams *et al.*, 1991), the exceptions being *E. dispar*, *E. faecalis*, *E. saccharolyticus* and *E. sulfureus*.

The Importance of Enterococci as Pathogens

Once thought of as harmless commensals, the importance of enterococci as pathogens - particularly in the nosocomial setting - is now much better appreciated than it was twenty years ago (Murray, 1998; Morrison *et al.*, 1997; Huycke *et al.*, 1998). According to one survey, enterococci were the second most common cause of infection (behind *Escherichia coli*, and ahead of better known pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*), responsible for 12% of nosocomial infections in the USA (Schaberg *et al.*, 1991). Additionally, the enterococci - particularly *E. faecium* (Morrison *et al.*, 1997; Huycke *et al.*, 1998; Moellering, Jr., 1998) - are important for their possession of intrinsic resistance to many antibiotics (e.g. cephalosporins, β-lactams and the aminoglycosides) and their ability to acquire resistance to practically all others currently available (e.g. tetracyclines, erythromycin, chloramphenicol, vancomycin) (Huycke *et al.*, 1998; Murray, 2000; Cetinkaya *et al.*, 2000).

The emergence and spread of enterococcal antibiotic resistance over the last decade has narrowed the already limited options for treatment of serious enterococcal infections to the extent that antibiotic failures are becoming commonplace (Landman and Quale, 1997; Swartz, 1994). More importantly, there is concern that multiply-resistant enterococci may serve as a pool of resistance genes for spread to other bacteria, such as the streptococci, staphylococci, *E. coli* and Listeria (Murray, 2000). Of particular concern is the potential for transfer of high level vancomycin resistance to methicillin-resistant *S. aureus* (MRSA) (Uttley *et al.*, 1988; Noble *et al.*, 1992).

Enterococcal infections range from urinary tract infections (UTI), infective endocarditis (IE), wound and soft tissue infections, to rare cases of meningitis and pneumonia (Moellering, Jr., 1995). *E. faecalis* is the predominant species isolated, accounting for between 80 and 90% of clinical enterococcal isolates. Of the remainder, most (5 to 15%) are *E. faecium*, which is considered a major pathogen chiefly because of its increasing antibiotic resistance (Huycke *et al.*, 1998; Moellering, Jr., 1995). UTIs are the most common enterococcal infection, with enterococci implicated in approximately 10% of all cases (Felmingham *et al.*, 1992) and up to 16% - making them the second most commonly isolated microorganism - amongst nosocomial cases (Schaberg *et al.*, 1991). The higher incidence in the latter setting has been attributed to the increased use of catheters, the existence of strong selective pressures for resistant enterococci in hospitals due to antibiotic overuse/misuse, and transmission of organisms between patients by contaminated hospital equipment and staff (Murray, 1990).

Next most common are wound infections (Megran, 1992), followed by bacteraemias (Moellering, Jr., 1998). A life-threatening infection of the heart, known as infective endocarditis (IE), can occur following enterococcal bacteraemias. Enterococci are the third most common cause of endocarditis, accounting for between 5 to 20% of cases (Watanakunakorn and Burkert, 1993; Megran, 1992; Simmons *et al.*, 1998).

Infective Endocarditis

As mentioned above, enterococci are the third most common cause of infective endocarditis. Most cases of enterococcal endocarditis are caused by *E. faecalis* with *E. faecium* being responsible for much of the remainder. The overall incidence of endocarditis in Europe has been variously quoted as ranging from 1.4 to 6.2 cases per 100,000 inhabitants (Hogevik *et al.*, 1995; Mylonakis and Calderwood, 2001). Despite advances in diagnosis and therapy, IE still results in considerable mortality and morbidity. A ten year study found an overall mortality of 21.4% (Watanakunakorn and Burkert, 1993), and mortality from enterococcal IE in particular ranges from 15 to 25% (Mylonakis and Calderwood, 2001). IE is believed responsible for over 200 deaths per year in England and Wales (Working Party Report of the British Society for Antimicrobial Chemotherapy, 1985). Hence, research into novel antibiotics, and into means of preventing microbial colonisation of the endocardium in the first place, is important. It is hoped that research into enterococcal endocarditis will lead to novel therapies that will circumvent current problems with antibiotic resistance.

IE commonly occurs in subjects with underlying structural cardiac defects who develop bacteraemia with certain bacteria, although there is an increasing proportion of cases in which no cardiac defects are obvious (Kaye, 1985a). It has been proposed that some form of endothelial damage (e.g. surgery) traumatises or removes endothelial cells, exposing components of the extracellular matrix, e.g. collagen, laminin and fibronectin; circulating platelets adhere to the area of damage and aggregate with fibrin and fibronectin, which become interwoven with the platelets. This vegetation, a non-bacterial thrombotic endocarditis (NBTE) grows as fibrin and platelets continue to bind. The presence of the NBTE predisposes to microbial colonisation during bacteraemia (e.g. following a urinary tract infection or surgery) (Johnson, 1993). Bacteria adhering to the NBTE become coated with more fibrous material and bacteria, which may serve to hide the organisms from circulating immune cells.

The heart valves are the most common site affected, with the left side of the heart being more commonly affected than the right. Other possible areas include mural endocardium or septal defects. Mitral valve infection is more common than aortic valve infection; the latter, in turn, is more common than combined aortic and mitral valve infection. Patients with prosthetic heart valves are at particular risk (Mylonakis and Calderwood, 2001).

Three groups of risk factors can be identified as predisposing to infective endocarditis: age, endothelial trauma and bacteraemia. The incidence of IE increases significantly with age (Kaye, 1985a), afflicting mainly elderly men (Mylonakis and Calderwood, 2001). The prevalence amongst elderly males has been attributed to the

increased number of genitourinary procedures performed - the genitourinary tract is the most common source of bacteraemia prior to enterococcal endocarditis (Megran, 1992). Mortality is significantly higher among the elderly (10.1% under the age of 60 years, 31.5% over 60 years (Watanakunakorn and Burkert, 1993)), and as the population of elderly people is growing in developed countries, endocarditis is assuming ever-increasing importance medically.

Predisposing conditions to endothelial trauma include congenital heart defects (which produce abnormally turbulent blood flow in the vicinity of the heart valve) (Fowler and Durack, 1994), atherosclerotic heart disease, mitral valve prolapse, hypertrophic obstructive cardiomyopathy and rheumatic heart disease (Mylonakis and Calderwood, 2001). Of these conditions, mitral-valve prolapse is the most common diagnosis predisposing to IE, with an incidence of approximately 100 cases per 100,000 patient-years (Bonow *et al.*, 1998). Prosthetic valve (or other cardiac) surgery also is a significantly predisposing condition, with prosthetic valve endocarditis accounting for between 7 to 25 % of IE cases in many developed countries (Berlin *et al.*, 1995).

Bacteraemia can occur after any surgical or dental procedure that damages a mucosal surface colonised by bacteria. Even eating or tooth brushing can result in transient bacteraemias that provide opportunities for bacteria to attach to and infect the endocardium (Strom *et al.*, 2000). Some dental procedures carry a sufficiently high risk of inducing IE that patients deemed at risk are given prophylactic antibiotic therapy. Bacteraemia can also occur when intravascular devices such as intravenous (IV) catheters, haemodialysis and hyperalimentation lines become infected,

introducing bacteria into the blood (Strom *et al.*, 2000; Fernandez-Guerrero *et al.*, 1995). One study of nosocomial cases on infective endocarditis found that at least half were the result of infected IV devices (Fernandez-Guerrero *et al.*, 1995).

IV drug abuse is a significant risk factor. In addition to inducing bacteraemia, the frequent injection of foreign particles can traumatise the myocardial endothelium (Kaye, 1985b). Most cases of IE in drug abusers are caused by *S. aureus* affecting the tricuspid valve on the right side of the heart, rather than the more common mitral valve (left side). However, this is made significant by the sheer number of cases accounted for by IV drug abusers (Lukes and Durack, 1993). The incidence in this group has been estimated at 150 to 2000 cases per 100,000 person-years, but can be higher in patients with known valvular disease (Frontera and Gradon, 2000).

Antimicrobial treatment of cases of enterococcal IE can be difficult, particular in cases where high-level multi-drug resistance is present. Generally, a prolonged course of a synergistic bactericidal combination of a cell-wall-active antimicrobial agent (e.g. ampicillin or vancomycin) plus an aminoglycoside is recommended as first-line treatment (Mylonakis and Calderwood, 2001). Unfortunately, optimal synergistic antimicrobial therapy is not possible where high-level resistance to both gentamicin and streptomycin is present (Murray, 2000), in which case susceptibility testing to determine MICs of other antibiotics takes on particular importance.

Enterococcal Antibiotic Resistance

Though not considered particularly virulent, enterococci are nevertheless considered important pathogens because of their antibiotic resistance. The enterococci come into contact with a wide range of other microorganisms in their gastrointestinal niche. Hence, as previously mentioned, the potential role of enterococci as reservoirs of antimicrobial resistance genes that may be transferred to more pathogenic species is a cause of particular concern. The enterococci are able to acquire and disseminate such genes via sex pheromone plasmids, broad host range plasmids and transposons (Murray, 1998).

The spread of high-level resistance to aminoglycosides, β-lactams and glycopeptides has greatly complicated the antimicrobial therapy of enterococcal infections. Low-level intrinsic resistance to β-lactams and aminoglycoside (attributed to low-affinity enterococcal penicillin binding proteins and to poor permeability of the cell wall, respectively) was not clinically problematic because a synergistic effect was obtained when these agents were used in combination. However, when high-level resistance to either β-lactams or aminoglycosides is present (the former a result of increased production of low-affinity penicillin binding proteins, the latter due to ribosomal alterations or enzymatic inactivation), the synergistic effects of such combination regimens are lost (Swartz, 1994). Today, up to 70% of enterococcal strains possess high level aminoglycoside resistance. The glycopeptide antibiotics vancomycin and teicoplanin have proven useful in the treatment of such enterococcal infections (Leclercq *et al.*, 1992). For many years, the glycopeptides

were virtually the only clinically-available drugs that could be consistently relied on to treat multidrug-resistant enterococci. However, glycopeptide resistance (caused by production of peptidoglycan precursors with reduced affinity for glycopeptides) has spread dramatically over the last decade. According to one survey, there was a 47% increase in infections caused by vancomycin-resistant enterococci (VRE) between 1994 and 1998 (Gerberding *et al.*, 1999). This has worsened the already difficult clinical problem of therapy of serious multidrug-resistant enterococcal infections such as endocarditis (Huycke *et al.*, 1998; Murray, 2000).

Fortunately, a number of newer drugs show some promise against multidrug-resistant enterococcal infections. For example, quinupristin-dalfopristin is highly effective against most strains of *E. faecium* (Caron *et al.*, 1997). However, it is poorly active against most strains of *E. faecalis* (Bhavnani and Ballow, 2000). Furthermore, while still relatively infrequent, instances of resistance are becoming increasingly widely reported (McNeil *et al.*, 2000; Rende-Fournier *et al.*, 1993). Linezolid is a member of the oxazolidinones, the first of a new class of orally-active synthetic antibiotics that possesses good activity against vancomycin-resistant *E. faecalis* and *E. faecium* (Diekema and Jones, 2001). Very few cases of enterococcal resistance have been reported thus far (Cetinkaya *et al.*, 2000; Diekema and Jones, 2001), but the fact that resistance has been reported at all serves as a warning that linezolid use needs to be carefully controlled.

Conjugative Sex Pheromone Plasmids

Sex pheromone plasmids are unique to, and widespread amongst, the enterococci (Wirth, 1994), and there are reports of detectable transfer *in vivo* (Hirt *et al.*, 2002).

Over one-third of *E. faecalis* clinical isolates carry pheromone response plasmids, whereas these plasmids are rarely found in *E. faecium* (Clewell, 1990). Antibiotic resistance can potentially be disseminated between enterococci at high frequency *in vivo* via such plasmids (Hirt *et al.*, 2002). Sex pheromone plasmids have also been known to mobilise other non-sex pheromone plasmids. Aside from genes encoding resistance to antibiotics, *E. faecalis* sex pheromone plasmids also commonly encode virulence determinants such as cytolysin (Gilmore *et al.*, 1994) and aggregation substance (Kreft *et al.*, 1992; Submuth *et al.*, 2000). Hence, the transfer of sex pheromone plasmids may also contribute to the spread or exchange of genes encoding virulence determinants between the enterococci and possibly other bacteria.

Recipient (plasmid-free) *E. faecalis* strains secrete multiple sex pheromones, each specific for a particular group of conjugative plasmids. The *E. faecalis* conjugative plasmids encode a mating response to their cognate sex pheromones. The mating response in donor (plasmid-containing) cells results in the synthesis of an aggregation substance, a surface protein able to bind to receptors present on the surfaces of both recipient and donor cells. Mating aggregates are formed between plasmid-containing donors with nearby recipient cells. Once a recipient cell has acquired a plasmid by conjugation, activity of the corresponding pheromone is curtailed via the secretion of modified, inactive forms (pheromone inhibitors) that competitively inhibit active pheromones. Secretion of unrelated pheromones specific for other plasmids continues uninterrupted (Dunny and Leonard, 1997). All known enterococcal sex pheromones, e.g. cAD1, cPD1, and cCF10, are chromosomally encoded and relatively hydrophobic, linear hepta- or octapeptides produced by

processing of the signal sequences of certain lipoprotein precursors (An and Clewell, 2002). Pheromone inhibitors are plasmid-encoded, in contrast, and synthesised as 20- to 23-amino-acid precursors which resemble lipoprotein signal sequences (An and Clewell, 2002).

Certain non-enterococcal species have been shown to produce pheromones which can induce *E. faecalis* sex plasmids, e.g. *S. aureus* produces a peptide which induces the plasmid pAM373 (Muscholl-Silberhorn *et al.*, 1997; Clewell *et al.*, 1985), suggesting a potential for exchange of genetic material with non-enterococcal species. Indeed, it is possible that pAM373 was responsible for the transfer of a transposon from *E. faecalis* to *S. aureus*, although the plasmid could not be maintained in the latter (Clewell *et al.*, 1985).

Enterococcal Virulence Factors

While antibiotic resistance is a major cause of concern with regards to enterococci and it doubtless contributes to enterococcal pathogenesis, antibiotic resistance by itself clearly does not account the prevalence of *E. faecalis* in enterococcal nosocomial infections. The fact that *E. faecium* is less susceptible to most commonly-used antibiotics suggests that *E. faecalis* possesses additional traits that may enhance its pathogenic potential. Consistent with this notion, *E. faecalis* strains have generally been found to harbour significantly more known virulence determinants than do *E. faecium* strains (Eaton and Gasson, 2001; Franz *et al.*, 2001). As enterococci are normal human commensals and not particularly virulent

pathogens, their virulence determinants can be difficult to pinpoint. Hence, current knowledge of enterococcal virulence factors is likely far from complete.

Recognised enterococcal virulence traits include adherence to host tissue, invasion and abscess formation, modulation of host inflammatory responses and secretion of toxic products. Enterococci are known to possess numerous surface-exposed proteins (antigens) which function as adhesins, the best known of which is aggregation substance. Adhesins enable adhesion of bacterial cells to various surfaces ranging from host tissue to other bacteria and are essential for colonisation and virulence. *E. faecalis* is renowned for its ability to exchange genes encoding antibiotic resistance efficiently by conjugation. Some enterococcal virulence factors are also similarly transmissible, e.g. aggregation substance and cytolysin production (Chow *et al.*, 1993; Hirt *et al.*, 2002). Several of the genes encoding virulence factors in *E. faecalis* have been cloned and the pathogenic effects of their products demonstrated in animal models or cultured cells, e.g. gelatinase, aggregation substance and cytolysin.

Aggregation Substance

E. faecalis sex pheromone plasmids commonly encode a surface protein termed aggregation substance (AS). Kreft *et al.* reported that AS expression increased *E. faecalis* adhesion of cells to cultured renal tubular cells (Kreft *et al.*, 1992), and increased uptake of AS-expressing cells into intestinal epithelial cells has also been demonstrated (Wells *et al.*, 2000; Olmsted *et al.*, 1994; Isenmann *et al.*, 2000). There is evidence that AS contributes to pathogenicity in rabbit endocarditis models by helping to increase the size of cardiac vegetations (Chow *et al.*, 1993; Schlievert

et al., 1998; Hirt *et al.*, 2002). The role of AS in promoting the growth of cardiac vegetations is likely to be related to recent evidence that AS promotes *E. faecalis* adhesion to fibrin (Hirt *et al.*, 2000) and to the extracellular matrix proteins fibronectin, vitronectin, collagen type I and thrombospondin (Rozdzinski *et al.*, 2001).

AS also seems to be responsible for promoting both the uptake of *E. faecalis* by polymorphonuclear leukocytes (PMNs) and macrophages and the subsequent intracellular survival of the bacterium in those phagocytes (Rakita *et al.*, 1999; Vanek *et al.*, 1999; Submuth *et al.*, 2000). Immune system evasion by hiding in PMNs and macrophages suggests a potential explanation for a recent report that antibodies against AS were not protective against rabbit endocarditis (McCormick *et al.*, 2001).

The expression of AS and the other plasmid transfer machinery is tightly-regulated *in vitro*. In contrast, Hirt *et al.* discovered that AS of pCF10 was induced *in vivo*, resulting in highly efficient *in vivo* plasmid transfer. Hence, they suggested that possession of AS and pCF10 might confer an *in vivo* survival advantage (Hirt *et al.*, 2002).

Cytolysin

Cytolysin is an *E. faecalis* haemolysin that is novel in its ability to function as a bacteriocin capable of killing a wide range of Gram-positive microorganisms (Gilmore *et al.*, 1994). In contrast, bacteriocins are usually narrow in spectrum, tending mainly to inhibit the growth of closely-related bacterial species (Jack *et al.*,

1995). This novel ribosomally-synthesised post-translationally-modified protein toxin is distantly-related to a group of bacteriocins termed the lantibiotics (Ike *et al.*, 1984). It consists of two subunits ($CylL_L$ and $CylL_S$) both of which are required for bactericidal activity (Gilmore *et al.*, 1994).

When present this toxin has been demonstrated to be an important virulence factor in cases of enterococcal infections. It has been shown to increase mortality rates in rabbit models of enterococcal endocarditis (Chow *et al.*, 1993). It has also been demonstrated to increase the severity and lethality of other enterococcal infections (Jett *et al.*, 1992; Ike *et al.*, 1984; Huycke *et al.*, 1991). One study associated the presence of cytolysin with a five-fold increase in mortality following *E. faecalis* bacteraemia (Huycke *et al.*, 1991). The pathogenic effects of cytolysin are thought to be the result of its alteration of leukocyte function, resulting in early, uncontrolled release of inflammatory agents. The consequent damaging of host tissue likely results in the release of nutrients to the benefit of the enterococci. Up to 60% of clinical *E. faecalis* isolates have been found to possess cytolysin (Ike *et al.*, 1987; Eaton and Gasson, 2001) compared with around 17% of strains from uninfected sources (Ike *et al.*, 1987) and 21% to 44% of strains isolated from food (Eaton and Gasson, 2001; Franz *et al.*, 2001). These findings suggest that selective pressures in the clinical setting may enrich for cytolysin.

Enterococcal Surface Protein (Esp)

Enterococcal surface protein (Esp) is a large, chromosomally-encoded (possibly transposon-encoded (Toledo-Arana *et al.*, 2001)) surface protein involved in enterococcal biofilm formation. It may also have a role in immune evasion. Shankar

et al. found that *esp* was present in 29% of *E. faecalis* blood isolates and 42% of endocarditis isolates but only in 3% of stool isolates, suggesting it an important role in pathogenesis (Shankar *et al.*, 1999). Another study discovered its presence in 57% of patient isolates, but did not provide further details about their ailments (Toledo-Arana *et al.*, 2001).

The exact role of Esp was unknown until very recently, when it was. reported that Esp was required for enterococcal biofilm formation (Toledo-Arana *et al.*, 2001). Complementation studies using *esp*-deficient strains revealed that Esp promoted primary attachment and subsequent biofilm formation on abiotic surfaces such as polystyrene and polyvinyl chloride from urine collection bags (Toledo-Arana *et al.*, 2001). It is likely that enterococcal biofilm formation on catheters and IV lines may play a role in enterococcal UTIs and bacteraemias, respectively. Likewise, enterococcal biofilms forming on heart valves following bacteraemia may have a significant a role in the formation and growth of vegetations in enterococcal endocarditis. Such biofilms would increase the difficulty of treating of enterococcal infections, as antimicrobial agents would have difficulty penetrating biofilms to reach bacterial cells buried deep within. Furthermore, the relative metabolic inactivity of deeply-buried cells also reduces their susceptibility to antimicrobial agents.

Esp possesses three major domains, the second of which is unusual in consisting of reiterations of distinct tandem repeating units. This domain is structurally similar to the Rib and C alpha surface proteins of group B streptococci, which have been shown to contribute to immune system evasion. Hence, it was hypothesised that the

N-terminal region was involved in adhesive interactions with host surfaces, while the central repeat region served to retract the N-terminal region from the surface in order to hide the protein from the immune system. Another possibility is that variations in the *esp* gene result in expression of alternative forms of the surface-localised Esp protein containing anywhere between three and nine tandem repeat units, effectively facilitating evasion of detection by the host immune system by a form of disguise (Shankar *et al.*, 1999).

Gelatinase

Gelatinase is an extracellular metalloendopeptidase encoded chromosomally by the gene *gelE*. The *gelE* and *sprE* (which encodes a serine protease) genes together form an operon (Qin *et al.*, 2000). Gelatinase hydrolyses gelatine, collagen, haemoglobin, and other bioactive compounds. Gelatinase production was found more frequently in hospital patients than in healthy volunteers (Coque *et al.*, 1995). Singh *et al.* reported its presence in 54% of *E. faecalis* endocarditis isolates, 58% of nosocomial clinical isolates, and 62% of nosocomial faecal isolates (Singh *et al.*, 1998a). In a mouse peritonitis model, non-gelatinase-producing strains had a slightly higher LD_{50} than gelatinase-producing strains but took significantly longer to exert a lethal effect compared with the wild-type (Singh *et al.*, 1998a; Qin *et al.*, 2000). The serine protease was also found to contribute to *E. faecalis* virulence in a similar manner (Qin *et al.*, 2000).

Extracellular superoxide

Some studies suggest that extracellular superoxide is an enterococcal virulence factor. Many *E. faecalis* strains and a few *E. faecium* strains have been found to

generate significant amounts of extracellular superoxide (Huycke and Gilmore, 1997; Huycke *et al.*, 1996). Comparison of *E. faecalis* endocarditis and bacteraemia isolates with commensal isolates from healthy volunteers revealed that extracellular superoxide production was 60% higher in the former (Huycke *et al.*, 1996). It is possible that *E. faecalis* utilises superoxide to damage host cells and thus gain the release of sequestered nutrients. Additionally, superoxide production enhanced *in vivo* survival of *E. faecalis* in mixed infection with *Bacteroides fragilis* in a subcutaneous infection model (Huycke and Gilmore, 1997), suggesting its use as a weapon against potential rivals. It has been demonstrated via a rat model that conditions exist in the mammalian intestinal tract that permit *E. faecalis* to generate hydroxyl radicals (Huycke *et al.*, 2001). These findings led Huycke *et al.* to suggest that *E. faecalis* may play a role in the pathogenesis of intestinal diseases such as colon cancer, which some have suggested may arise through oxidative damage. Subsequently, they were able to demonstrate using a rat model that hydrogen peroxide arising from dismutation of extracellular superoxide was responsible for damaging colonic epithelial cells. Specifically, increased DNA damage was found in the lumenal cells, supporting the notion that extracellular free radical production by *E. faecalis* in promoting chromosomal instability associated with polyps and colorectal cancer (Huycke *et al.*, 2002).

Ace

Adhesion to eukaryotic extracellular matrix proteins (ECM) such as the collagens, proteoglycans, fibronectin and laminin may play a crucial role in the pathogenesis of many bacterial infections (Westerlund and Korhonen, 1993). An *E. faecalis* gene, *ace*, has been identified that encodes an adhesin similar to the collagen binding

protein Cna of *S. aureus* (Rich *et al.*, 1999). This protein was demonstrated to be responsible for *E. faecalis* binding to the extracellular matrix proteins collagen types I and IV and laminin (Nallapareddy *et al.*, 2000a; Nallapareddy *et al.*, 2000b). Analysis of sera from patients with *E. faecalis* infections, and in particular those with *E. faecalis* endocarditis, revealed that Ace was commonly expressed *in vivo*. In contrast, expression occurred mainly at 46°C *in vitro*, with little expression evident at 37°C (Nallapareddy *et al.*, 2000a). These findings suggest that Ace may play a role in *E. faecalis* pathogenesis.

EfaA

Guzman *et al.* had previously demonstrated that growth in serum increased the ability of *E. faecalis* to adhere to heart cells (Guzman *et al.*, 1989; Guzman *et al.*, 1991). Studies of the antigenic composition of the cell wall of endocarditis-causing *E. faecalis* strains revealed a dominant 37 kDa antigen specific recognised by sera from patients with infective endocarditis (Aitchison *et al.*, 1987). This antigen was strongly expressed after growth in Brain Heart Infusion medium supplemented with 1% serum, but not after growth in chemically-defined medium (Lambert *et al.*, 1990). The endocarditis-specific expression of the 37 kDa antigen was subsequently confirmed and exploited in a serodiagnostic ELISA, which successfully identified cases of *E. faecalis* endocarditis and was able to discriminate such cases from endocarditis due to other streptococci, and from *E. faecalis* infections other than endocarditis such as those in the urinary tract (Shorrock *et al.*, 1990). The 37 kDa antigen has since been cloned, sequenced and designated EfaA (Lowe *et al.*, 1995) and its possible function as a virulence factor will be discussed further below.

The Cluster Nine Family of ABC Transporters

EfaA is homologous to a group of streptococcal surface proteins originally identified as adhesins. This group was initially designated the lipoprotein receptor antigen I (LraI) family of polypeptides (Jenkinson, 1994); members have been identified in many species of streptococci (Berry and Paton, 1996; Jenkinson, 1994; Viscount *et al.*, 1997; Correia *et al.*, 1996; Janulczyk *et al.*, 1999; Kitten *et al.*, 2000) and in at least two species of enterococci (Lowe *et al.*, 1995; Singh *et al.*, 1998b). They are lipid-modified at their N-terminals and characterised by possession of the consensus sequence Leu-X-X-Cys near this terminal (Jenkinson, 1994).

ABC-type transporters are a superfamily of extracytoplasmic receptor-dependent transporters found mainly in prokaryotes (Higgins, 1992). They are so named because they harness the energy of ATP hydrolysis to transport substrates across membranes against concentration gradients. In *E. faecalis*, *Streptococcus gordonii*, *Streptococcus parasanguis*, *Streptococcus pneumoniae* and many other microorganisms, the LraI polypeptide is one component of an ATP-binding cassette (ABC)-type transporter (also referred to as ABC permeases or traffic ATPases) (Fenno *et al.*, 1995; Kolenbrander *et al.*, 1994; Lowe *et al.*, 1995; Dintilhac and Claverys, 1997a). These transporters are in fact the Gram-positive equivalent to the periplasmic binding protein-dependent transport systems found in Gram-negative bacteria.

It is believed that the LraI polypeptides function in the ABC protein complex as solute binding-protein receptors (or extracytoplasmic receptors), binding specific

substrates which are subsequently translocated across the cytoplasmic membrane through a channel formed by hydrophobic transmembrane proteins (HMP). ATP-binding proteins (ATPases) located at the cytosolic face of the membrane provide energy for the translocation process by coupling hydrolysis of ATP to transmembrane solute transport. Eight families of bacterial solute-binding proteins had been defined previously (Tam and Saier, Jr., 1993), but since the LraI-containing ABC complexes showed no homology to any of those existing families, they were designated as cluster 9 (Dintilhac and Claverys, 1997a).

There are now also suggestions that in addition to adhesion and solute transport, certain ABC transporters are involved in activities such as initiation of signal transduction (e.g. the Pst system of *E. coli*), chemoreception and regulation of cellular functions such as the induction of competence, e.g. the Ami-AliA-AliB oligopeptide ABC transporter also appears involved in transformation (Alloing *et al.*, 1996). These findings suggest that there is still much to be learned about the cluster 9 family.

The ATPase

In the typical ABC transporter, the ATPase usually consists of two different domains (Higgins, 1992), each of which is an ATP-binding protein about 200 amino acids long anchored to the cytoplasmic face of the membrane via a single transmembrane helix (Tam and Saier, Jr., 1993). As with the transmembrane domains, it is likely that the ATP-binding domains function as dimers (Higgins, 1992).

ClustalW analysis revealed that EfaC is highly homologous to its cluster 9 counterparts such as PsaC (*S. pneumoniae*, 47% identical, FimC (*S. parasanguis*, 53% identical, ScaC (51% identical) and EfmC (*E. faecium*, Genbank, Accession Number AF097414, 60% identical). There is also considerable sequence identity between the ABC-binding domains of cluster 9 ABC permeases and that of other ABC transporters. This is a distinguishing feature of members of the ABC transporter superfamily which separates them from other nucleotide-binding proteins. Two short motifs known as Walker motifs A (consensus GXXGXGKST) and B (consensus hhhhDEPT where h is any hydrophobic amino acid) are believed to constitute the ATP-binding site. The aspartate residues of these motifs bind Mg^{2+}, which is required for ATPase activity and correct positioning of ATP for catalysis. The "switch" region (consensus xGh) at the C-terminal end is thought to be involved in signal transport following hydrolysis of ATP (Schneider and Hunke, 1998). There is also a glutamine-glycine-rich linker peptide that may act as a signal transducer; it is a signature sequence unique to the ABC family (Schneider and Hunke, 1998). All these features are present in both EfaC and EfmC.

The Hydrophobic Membrane Protein

In the typical ABC transporter, the transmembrane component consists of one, or more usually two, hydrophobic domains/proteins. These domains form a channel through which the substrate crosses the membrane, and are believed to contribute a substantial degree of substrate specificity. The transportation process is powered by energy provided by the ATPase components.

In most transporters, each transmembrane domain consists of six highly hydrophobic membrane-spanning α-helical regions, most transporters having twelve membrane spanning regions in total (the "two-times-six" paradigm) (Higgins, 1992). In line with this model, analysis of potential transmembrane helices in ScaB (Kolenbrander *et al.*, 1994), FimB (Fenno *et al.*, 1995) and PsaC (Novak *et al.*, 1998) revealed that they possessed six hydrophobic α-helical transmembrane regions exhibiting a regular periodicity and a very hydrophilic C-terminal tail. As only one hydrophobic domain is encoded by the typical cluster 9 operon, it is likely that their transmembrane domains function as dimers.

EfaB of *E. faecalis* is highly homologous to its cluster 9 counterparts PsaB (*S. pneumoniae*, 57% identical), FimB (*S. parasanguis*, 54% identical), Sca (55% identical) and EfmB (*E. faecium*, Genbank, Accession Number AF097414, 68% identical).

The Solute Binding Protein

LraI Lipoproteins and Cell Adhesion

The first LraI lipoproteins were discovered as prominent surface antigens (Lowe *et al.*, 1995; Andersen *et al.*, 1993; Russell *et al.*, 1990) and were originally described as adhesins. *S. gordonii* ScaA was implicated in co-aggregation with human oral actinomyces (Andersen *et al.*, 1993). FimA of *S. parasanguis*, suggested to be a major virulence factor determinant associated with initial colonisation of damaged

cardiac tissue, appeared to be located on the tips of fimbriae (Fenno *et al.*, 1995). The addition of purified FimA appeared to reduce adhesion of *S. parasanguis* to saliva-coated hydroxyapatite (SCHA) (Oligino and Fives-Taylor, 1993) and *S. parasanguis fimA* mutants seemed to display reduced binding to fibrin monolayers and decreased ability to cause endocarditis in a rat model (Burnette-Curley *et al.*, 1995). *S. pneumoniae psaA* mutants exhibited significantly reduced adherence to type II pneumocytes (A549 cells) and were much less virulent than their wild-type counterparts in intranasal or intraperitoneal challenge of mice (Berry and Paton, 1996). Lmb was reported to mediate the attachment of *Streptococcus agalactiae* to human laminin (Spellerberg *et al.*, 1999). These and other reports were generally taken as evidence that cluster 9 proteins played a role in cell adherence.

One possible explanation for the adhesive properties of the LraI lipoproteins is that it is a by-product of their putative role as the solute-binding components of ABC permeases. Binding to metal ions complexed to and hence immobilised on the surfaces of host tissue membranes or other bacterial cells results in adherence to those surfaces. If so, LraI polypeptides would be attractive targets for novel antimicrobial therapies – anti-LraI vaccines could potentially prevent or be used to treat infections such as infective endocarditis (Oligino and Fives-Taylor, 1993).

However, not all LraI lipoproteins have been shown to be associated with adhesion, notably ScbA (from *Streptococcus crista* CC5A) (Correia *et al.*, 1996). Also, Gram-negative cluster 9 LraI counterparts are periplasmic, and therefore most unlikely to be adhesins. A model of PsaA derived from crystallisation studies suggests that if the molecule is lipid-anchored to the bacterial membrane, its size would prevent it

protruding beyond the cell wall and surrounding polysaccharide capsule of *S. pneumoniae*. Instead it would be encased beneath the peptidoglycan/teichoic acid cell wall (Lawrence *et al.*, 1998). Electron micrographs of *Staphylococcus epidermidis* suggest a similar location for the PsaA homologue SitC (Cockayne *et al.*, 1998). Hence, it is possible that the loss of host adhesion observed in PsaA knockout mutants could be indirectly caused by a secondary protein being rendered absent or non-functional via metal deprivation. Furthermore, evidence that Gram-positive lipoproteins can be released into the environment (Sutcliffe and Russell, 1995) suggests an alternative explanation to surface exposure for the apparent antigenicity of some of the LraI lipoproteins. In short, it is starting to appear more likely that LraI lipoproteins have an indirect role in adhesion.

Structural Features of the Cluster 9 Solute Binding Proteins

EfaA of *E. faecalis* is highly homologous to its cluster 9 counterparts PsaA (*S. pneumoniae*, 61% identical), FimA (*S. parasanguis*, 63% identical), ScaA (52% identical) and EfmA (*E. faecium*, 63% identical, Genbank, Accession Number AF097414 (Flatman, 1999)). Polypeptides of this family generally have a molecular mass between 32 to 35 kDa. The typical protein is approximately 280 to 340 amino acid residues long, and four distinct regions can been identified (Jenkinson, 1994).

The first region, consisting of the first 32 amino acids of the precursor polypeptide, is the least well conserved; it includes a hydrophobic 19- or 20-residue leader sequence thought to direct transmembrane transport of the protein. The C-terminal end of the leader sequence (residues 17 to 20 in ScaA) contains a L,S/A,A/G,↓,C,S,G motif - this is the cleavage site for the enzyme signal peptidase

II. Cleavage of this leader sequence produces the mature protein. With Gram-positive homologues, the N-terminal cysteine of the processed polypeptide is lipid-modified – it is bound to palmitic acid in the cell membrane via an acyl-glyceride bond (Jenkinson, 1992), anchoring it to the cytoplasmic membrane.

In contrast, the Gram-negative homologues are not lipidated and are periplasmic in location. Since Gram-positive bacteria lack an outer membrane, tethering of the lipoprotein to cell membrane is presumably functionally equivalent to periplasmic localisation (Sutcliffe and Russell, 1995), serving to prevent loss of lipoprotein to the environment and/or to maintain its position relative to other components of the ABC transporter. Such an explanation would make sense, for example, in the case of TraC (a lipoprotein, though not a cluster 9 homologue) of *E. faecalis*, which is believed to be a receptor for the peptide sex pheromone cAD1 (Tanimoto *et al.*, 1993). Lipoproteins can nevertheless be released from the cell surface in an acylated form, as illustrated by the recovery of proteins from culture supernatants of *Streptococcus mutans* (Sutcliffe *et al.*, 1993). Some lipoproteins appear to be released from the bacterial cell depending on the phase of growth and lipoprotein enzymes can be released via proteolytic cleavage. Whether the release of lipoproteins from the cell is widely exploited as a mechanism for regulating their activity at the cell surface or is simply a consequence of general cell turnover is unclear (Sutcliffe and Russell, 1995). Whether Gram-positive cluster 9 lipoproteins in particular are released into the supernatant is also unclear.

The second (residues 29/32 to 146/148) and fourth (residues 196/198 to 308/310) regions are largely β-structure, and hence termed B1 and B2, respectively. The third

region (residues 147/149 to 195/197), known as the α-region form, is an α-helix which bridges the β-sheets. Most of the dissimilarity is found in the region of the signal peptide, which is not part of the mature protein. The α-region contains the next most dissimilar sequence (a segment of about 40 residues). Rather than a single α-helix, the corresponding regions of ABC-type binding proteins belonging to clusters 1 to 8 contain pairs of α/β/α domains which are similar, but more elaborate. In these other families the polypeptide makes two or three inter-domain crossovers. These crossover (or hinge) polypeptide segments have an extended conformation and allow movement of one domain with respect to the other, and the resulting conformational change is believed to be essential for recognition of the ligand-complexed binding proteins by their respective ABC-type permeases (Quiocho and Ledvina, 1996). This requirement of conformational change for recognition and lack of "hinge" peptides led Lawrence *et al.* to suggest that conformational change in cluster 9 proteins must be of a different structural nature to that observed in other clusters, implying that cluster 9 is evolutionarily distant from the other ABC-type systems (Lawrence *et al.*, 1998). The B1 and B2 sequences are relatively well-conserved, with the B1 region the more so (residues 125-148 being the overall best conserved) (Jenkinson, 1994), consistent with previous observations that N-terminal domains of solute binding proteins in general are better conserved than C-terminal domains (Higgins, 1992).

The solute binding site is located at the interface between the two B domains. Two-domain structures are typical of the solute binding proteins, probably because solute binding imposes constraints on, and hence defines to a large extent, receptor structure. Crystallographic studies revealed that the metal binding site of PsaA

comprised the side chains of H67, H139, E205 and D280 (Lawrence *et al.*, 1998). The metal bound was interpreted to be zinc. Subsequently, Lee *et al.* pointed out that the geometry of the PsaA binding site was consistent with the binding of hexacoordinated Mn^{2+} via a bidentate interaction with E205 (Lee *et al.*, 1999).

A relationship between ligand specificity and receptor homology has been observed (Higgins, 1992; Tam and Saier, Jr., 1993). Considering the high degree of homology amongst cluster 9 solute binding proteins even amongst unrelated (e.g. in Gram-negative and Gram-positive) bacteria, it is likely that all bind similar substrates. Hence, as will be discussed below, Dintilhac *et al.* tentatively assigned substrates for each of the various proteins on the basis of sequence similarity to those whose substrates were known already (Dintilhac *et al.*, 1997b).

Solute Binding Protein Substrates – Divalent Cations

Prokaryotic ABC transporters are involved in the import of substances ranging from sugars, amino acids, opines, metal cations to vitamins. However, a general correlation between the relatedness of binding proteins and the nature of solute bound was known to exist (Higgins, 1992). The first hints that cluster 9 operons might encode metal permeases came in 1995, when *Synechocystis* sp. cluster 9 *mntA* mutants were found to have dramatically reduced growth rates which could be restored by manganese supplementation in the growth medium. Furthermore, the *Synechocystis* sp. photosystem II complex, in which Mn^{2+} plays an essential role in oxygenic photosynthesis, is defective in *mntA* mutants. Hence, Bartsevich and Prakasi concluded that *mntABC* constituted a manganese permease (Bartsevich and Pakrasi, 1995). Then, Dintilhac *et al.* discovered that the addition of zinc to

S. pneumoniae adc mutants restored growth and near-normal spontaneous transformation, whereas extraneous manganese was absolutely required for growth of *S. pneumoniae psa* mutants, leading them to propose Adc and Psa being high affinity ABC Zn^{2+} and Mn^{2+} permeases, respectively (Dintilhac *et al.*, 1997b).

Via phylogenetic studies, divalent cationic ligands were tentatively assigned to other members of the cluster 9 family. For example, AdcA protein contains a histidine-rich tract, HDHGEEGHHH, thought to be responsible for binding zinc (histidine tags were known or thought to be part of Ni^{2+}-, Cu^{2+}- and Zn^{2+}-binding sites). Since the cluster 9 members ZnuA, PZP1 and Syn9 all contain histidine-rich regions located at the very same position as in AdcA, they were proposed to be Zn^{2+}-binding receptors. Similarly, because of the high relatedness of EfaA, FimA, ScaA, ScbA, PsaA, Spy9, SsaB and Step to PsaA, these were proposed to be manganese-binding (Dintilhac *et al.*, 1997b).

Since then, Sca of *S. gordonii* has been demonstrated to be a high affinity ABC Mn^{2+} permease via uptake experiments involving $^{54}Mn^{2+}$ (Kolenbrander *et al.*, 1998). Numerous similar findings from investigations into metal requirements of mutants of other cluster 9 transporters have also been published, e.g. Mnt of *Synechocystis* 6803 (Bartsevich and Pakrasi, 1996) was shown to transport Mn^{2+} whereas Znu of *E. coli* (Patzer and Hantke, 1998) and PZP1 of *Haemophilus influenzae* are Zn^{2+} transporters. Slot blot experiments by Janulczyk *et al.* initially indicated that the *Streptococcus pyogenes* solute binding protein might be unusual in that MtsA bound Zn^{2+}, Cu^{2+} and Fe^{2+} cations (Janulczyk *et al.*, 1999). However, because heterologous expression of proteins such as the iron protein rubredoxin in

E. coli had resulted in zinc-substituted proteins, Claverys suggested that the MtsA results were in fact anomalous and a product of the expression of MtsA in the heterologous host *E. coli* (Claverys, 2001). On the basis of sequence alignments of solute binding proteins and following an extensive analysis of reports, taking into consideration sequence homologies, permease mutant metal requirements, metal binding, metal uptake and metal-dependent regulation, Claverys proposed that the cluster 9 family in fact consisted at least 47 members divisible into two subclusters specific for either Zn^{2+} or Mn^{2+} (Claverys, 2001). In this work, these subclusters will be referred to as "Adc-like" or "Psa-like", respectively. It also emerges that what had hitherto been referred to as the LraI family in fact best corresponded to a Gram-positive subset of solute-binding proteins that had been shown or were predicted to bind Mn^{2+} (Claverys, 2001).

To add to differences in substrate specificities and sequence homology (in particular the His-rich tract) compared with the Psa-like homologues, Adc-like homologues have also been shown to be differently regulated. Psa-like homologues appear to be regulated by DtxR-like proteins (discussed below). On the other hand, Adc expression is regulated by AdcR, which belongs to a newly-discovered family of metalloregulators with some homology to *E. coli* MarR (AdcR and MarR are 25% identical) (Claverys, 2001) while the Adc-like Ycd and Znu of *B. subtilis* and *E. coli* are regulated by Zur, a zinc-responsive Fur homologue (Patzer and Hantke, 1998; Gaballa and Helmann, 1998; Patzer and Hantke, 2000). We have identified in the unannotated *E. faecalis* V583 genome available at the website of the Institute for Genomic Research (TIGR) two Psa-like and two Adc-like homologues, three MarR-like proteins and a Zur homologue (unpublished observations).

The form of metal ions transported by cluster 9 ABC-type permeases was unknown, as the above studies did not present any evidence for direct binding and transport of metal ions. In apparent contradiction to the studies demonstrating Mn^{2+} transport by *S. pneumoniae* Psa, in a study of the crystal structure of PsaA, it appeared that Zn^{2+} rather than Mn^{2+} was bound (Lawrence *et al.*, 1998). This raised the possibility that perhaps Mn^{2+} was being transported by Psa in the form of chelated complexes rather than being directly bound and transported as free ions. Certainly, uptake of Mn^{2+} by the P-type ATPase of *Lactobacillus plantarum* is known to be stimulated by citrate and other tricarboxylic acids, although citrate itself did not appear to be imported (Hao *et al.*, 1999; Archibald, 1986). It has generally been observed that cluster 9 permeases are not the only metal permeases present in most bacteria, as inactivating cluster 9 transporters did not completely abolish uptake of cations (Kolenbrander *et al.*, 1998; Dintilhac *et al.*, 1997b; Bartsevich and Pakrasi, 1996; Bearden and Perry, 1999).

It is important to note that in no case were any experiments performed that demonstrated unequivocally that cluster 9 protein complexes actually transported those metal ions. Simple binding by solute binding proteins does not demonstrate that the metals are then passed through the hydrophobic transmembrane protein, so evidence from solute binding protein mutants cannot be considered one hundred percent conclusive. Such mutations may affect the activity of heterologous ion channels transporting zinc or manganese (i.e. the solute binding proteins could be passing bound metal ions to channels other than their cognate ones). Similarly, HMP or ATPase mutations may indirectly affect the working of other channels. Studies of

substrate transport involving reconstituted transport systems *in vitro* would provide indisputable evidence but designing such experiments would be very difficult at best.

The Role of Divalent Transition Metal Cations in Bacteria

The importance of Fe^{2+} is well documented for many pathogens (Crosa, 1997). Iron is easily oxidised to insoluble ferric hydroxide in solution, making it unavailable to bacteria. In the human host, further iron limitation by sequestration into cells serves as a primary host defence mechanism. Iron-scavenging strategies are therefore crucial to bacterial colonisation and growth - i.e. survival. Hence, most bacteria, particularly Gram-negative species, possess multiple iron-acquisition mechanisms, such as siderophores, extracellular lactoferrin and transferrin binding proteins, and haemolysins. Many of these mechanisms are inducible, e.g. the *yfe* operon of *Yersinia pestis*; iron limitation inactivates regulatory proteins, resulting in derepression of iron-acquisition and other virulence genes.

The essential nature of Fe^{2+} in promoting growth of Gram-negative pathogens is well established, but little is known about the involvement of Fe^{2+} and other redox-active ions such as Mn^{2+} in Gram-positive infections. There is evidence that Mn^{2+} can support the growth of streptococci under Fe^{2+}-limited conditions. There are a few exceptional species which appear to require almost no iron, notably members of the family *Lactobacillaceae* (Weinberg, 1997), the Lyme disease pathogen *Borrelia burgdorferi* (Posey and Gherardini, 2000) and *Treponema pallidum* (Posey *et al.*, 1999). Such bacteria have an obligate requirement for manganese, having evolved metabolic and survival strategies using manganese rather than iron. The ability to do

without iron may confer a selective advantage where there is intense competition for limited iron. However, such microorganisms may be unable to carry out certain reactions. For example, aerobic respiration is not possible without cytochromes, which in turn require iron.

Zinc is an important cofactor for a variety of enzymes and has a structural role in many proteins (Suhy and O'Halloran, 1996). It plays an important role in competence regulation, probably during transcription of competence genes. Deprived of zinc, *S. pneumoniae* cells were unresponsive to competence stimulating peptide (Dintilhac *et al.*, 1997b). Similar to iron, Zn^{2+} is an element that, depending on the concentration, can be an essential micronutrient. However, it can also be toxic, interfering with vital functions by competing with other metal ions for biologically important ligands such as active sites of enzymes and transport proteins (Westenberg and Guerinot, 1997). Hence, intracellular levels of zinc must be precisely regulated.

Manganese is a trace element required for the normal growth of most organisms. Numerous and diverse activities require manganese, e.g. sporulation in sporulating bacteria and production of numerous secondary metabolites, antigens and toxins (Jakubovics and Jenkinson, 2001). Examples of manganese-cofactored enzymes involved in basic metabolism include pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Mn^{2+} is also essential for other metabolic pathways, e.g. the photosystem II pigment-protein complex of *Synechocystis* sp. requires a Mn^{2+} ensemble for oxygenic photosynthesis (Bartsevich and Pakrasi, 1995). There are enzymes which specifically require Mn^{2+} for activity, including

manganese-cofactored superoxide dismutase (MnSOD) and mangani-catalase (Christianson, 1997). Some Gram-positive endospore-forming bacteria (e.g. *B. subtilis*, *Bacillus megaterium*, *Clostridium perfringens*) require an Mn^{2+}-activated 3-phosphoglycerate mutase (PGM) for glycolysis (Chander *et al.*, 1998). The Mn^{2+} association has been shown to be pH-dependent, and it was postulated that this pH-sensing by Mn^{2+} represented a regulatory mechanism (Kuhn *et al.*, 1995). Other strictly Mn^{2+}-dependent enzymes such as arginase and PEP carboxykinase may utilise similar regulatory mechanisms (Kuhn *et al.*, 1995). Mn^{2+} has an important role in *E. coli* signal transduction, whose PrpA and PrpB proteins are homologous to a family of eukaryotic Mn^{2+}-containing serine/threonine protein phosphatases widely found known to modulate complex signalling pathways (Barford, 1996). Mn^{2+} also has important non-enzymatic roles, e.g. it has structural roles in the stabilisation of bacterial cell walls and in certain bacterial products such as some secreted antibiotics (Archibald, 1986), and is also thought to be involved in non-enzymatic detoxification of reactive oxygen species. Notably, some lactic acid bacteria incorporate high levels of intracellular Mn^{2+} as a protectant in place of enzymatic SOD (Archibald, 1986). The precise mechanism by which non-enzymatic Mn^{2+} detoxifies reactive oxygen species in microorganisms is not understood. Experiments such as one in *B. subtilis* which demonstrated that the supplementation of growth medium with Mn^{2+} was able to substitute for SOD activity in SOD-deficient mutants suggest that Mn^{2+} may play a similar role in other bacteria, albeit to a lesser extent than may be the case in the lactococci (Inaoka *et al.*, 1999).

As described above, there is substantial evidence of a role in bacterial pathogenesis for members of the cluster 9 family of putative divalent metal transporters. Recent findings, e.g. the up-regulation of expression of the *S. gordonii* Sca complex under Mn^{2+}-limiting conditions (Jakubovics *et al.*, 2000), suggest that the cluster 9 proteins may play an essential role in Mn^{2+} homeostasis, and that the pathogenic effects are an indirect consequence of this role. High affinity uptake mechanisms for of Mn^{2+} may be vital for the survival of bacteria in mammalian hosts which may impose Mn^{2+}-limitation as a form of self-defence. In the human host, serum Mn^{2+} levels are typically around 20 nM (Krachler *et al.*, 1999) as most available Mn^{2+} is complexed with albumin and transferrin. This presents a problem for growth of the organism, necessitating specialised systems to acquire this trace metal. Hence, it has been suggested that the Sca transporter may be a Mn^{2+}-scavenging system (Kolenbrander *et al.*, 1998), with adhesion properties occurring as a by-product of this role.

Mn^{2+} is highly soluble and unlike iron does not catalyse hydroxyl radical formation (Cheton and Archibald, 1988). However, because excess metal cations can be toxic, Mn^{2+} accumulation must be under tight control. Most bacteria are known to possess manganese uptake transporters that are activated or repressed in response to metal ion availability. Two families of bacterial manganese-responsive transcriptional regulators have been discovered and shown to be present across the eubacteria (Posey *et al.*, 1999; Que and Helmann, 2000; Jakubovics *et al.*, 2000). *T. pallidum* possesses a Mn^{2+}-dependent transcriptional repressor termed TroR, a homologue of DtxR of *Corynebacterium diphtheriae* (Posey *et al.*, 1999). Since internal Mn^{2+} levels in the human host vary from 0.08 to 0.13 μg/ml in the blood to 9 or 10 μg/ml in the central nervous system, Posey *et al.* have proposed that the TroR regulatory

system of *T. pallidum* may serve as a means of preventing intracellular Mn^{2+} rising to toxic levels as it migrates to various sites within the host (Posey *et al.*, 1999).

Genetic Organisation of the Psa-like Cluster 9 Operon

Each of the three components of the ABC permease is encoded by its own gene, and the three genes are organised in tricistronic operons in which each gene is present in single copy. Usually, but not always, the first gene encodes the ATP-binding protein, the second the hydrophobic membrane protein, and the third the LraI lipoprotein (Fenno *et al.*, 1995; Kolenbrander *et al.*, 1994; Lowe *et al.*, 1995; Dintilhac and Claverys, 1997a). In operons organised in this manner, the ATPase (including the stop codon) and HMP genes typically overlap, with the overlap ranging from 4 bp in the *E. faecalis*, *E. faecium*, *S. mutans*, *S. parasanguis*, and *S. pneumoniae psa* operons to 8 bp in the *S. pneumoniae adc* operon. The distance between the HMP and lipoprotein genes is more variable, ranging from overlaps of 4 bp, 14 bp and 38 bp in *S. epidermidis*, *E. faecalis* and *E. faecium*, respectively, to separations of 9 bp in *S. pneumoniae* and up to 47 bp for *S. mutans*. Genes for other proteins may also be present in the operon. *S. parasanguis* (Fenno *et al.*, 1995), *S. pneumoniae* (Novak *et al.*, 1998), *S. gordonii* (Kolenbrander *et al.*, 1994; Jakubovics *et al.*, 2000), *S. crista* (Correia *et al.*, 1996) and some other microorganisms possess a gene encoding a thiol peroxidase homologous to that in *E. coli* downstream of their lipoprotein genes. Surprisingly, in *S. mutans*, the gene downstream of the lipoprotein was not a peroxidase but rather a protein homologous to the iron-dependent repressor DtxR from *C. diphtheriae* (Kitten *et al.*, 2000; Spatafora *et al.*, 2001).

A promoter precedes the first (ATPase) and fourth (where present) genes but neither the HMP or lipoprotein genes have their own promoters - both are transcribed by read-through from the first gene, as demonstrated by the lack of FimA production in the *S. parasanguis* VT952 mutant, in which the *fimB* gene upstream had been interrupted by an insertion mutation (Fenno *et al.*, 1995). Potential ribosome binding sites (RBS) are located upstream of all three genes. Immediately downstream of the third gene is a palindromic sequence followed by a run of several adenines which is probably a rho-independent terminator (Fenno *et al.*, 1995; Kolenbrander *et al.*, 1994), resulting in a polycistronic mRNA transcript approximately 2.5 kb long in the case of the *E. faecalis efa* tricistronic operon. However, in operons containing a fourth gene, the presence of this weak transcription terminator does not necessarily prevent read-through to that gene, resulting in polycistronic mRNA approximately 3.2 kb long. The "leakiness" of this terminator appears to vary from species to species. In *S. gordonii*, which has a thiol peroxidase *tpx* gene immediately downstream of the *scaCBA* genes, the 2.6 kb *scaCBA* transcript was the major transcript with only a small amount of 3.2 kb readthrough transcript being produced (Jakubovics *et al.*, 2000). In contrast, in *S. pneumoniae*, only read-through 3.1 kb transcript including the downstream thiol peroxidase *psaD* (*tpx*) gene was found (Novak *et al.*, 1998). It should be noted that where experiments have been conducted, e.g. in *S. gordonii*, transcription of the fourth gene from its own promoter (as opposed to through read-through) has been found to be regulated independently of the ABC transporter promoter (Jakubovics *et al.*, 2000).

With many ABC-type permeases, the solute-binding protein is produced in excess of the membrane-integral and ATP-binding cytoplasmic components (Podbielski and

Leonard, 1998; Podbielski *et al.*, 1996). The organisation of the *S. pyogenes mtsABC* operon is atypical of the cluster 9 family in that the first gene of the operon encodes the lipoprotein MtsA. A leaky transcriptional terminator between *mtsA* and *mtsB* allows the MtsA lipoprotein transcript to be expressed in 10- to 20-fold excess over the polycistronic transcript (Janulczyk *et al.*, 1999). A similar regulatory mechanism is not possible in the other cluster 9 operons where the lipoproteins encoded at the 3' end of the operons, but the possibility remains that translational controls may modulate the relative amounts of permease components.

Genes encoding DtxR-like proteins have been discovered either upstream or downstream of the *S. epidermidis* and *S. mutans* cluster 9 operons, respectively (Hill *et al.*, 1998; Kitten *et al.*, 2000; Spatafora *et al.*, 2001). Such findings have not been universal, but a number of cluster 9 operons have since been demonstrated to be regulated by DtxR-like proteins, as will be discussed below.

Metal-Dependent Regulation of Expression

Prokaryotic metal cation homeostasis is generally achieved by regulation of transcription of genes encoding metal cation uptake proteins. Metal-responsive transcriptional regulators sense the intracellular levels of their cognate cations and modulate transcription of their regulons accordingly. Two main families of metalloregulators responsive to the divalent transition metal cations Fe^{2+}, Mn^{2+} and/or Zn^{2+} have been recognised in bacteria, the paradigms of which are *E. coli* Fur (ferric uptake repressor) and *C. diphtheriae* DtxR (diphtheria toxin repressor). The

DtxR family includes regulators of iron and manganese transport (Que and Helmann, 2000).

The Fur Family of Metalloregulators

The coordinated expression of virulence genes in response to iron deprivation is a well known phenomenon in Gram-negative bacteria. Iron-responsive gene expression is under the control of an iron-binding transcriptional repressor, of which *E. coli* Fur is the classic example. Members of the Fur family are known to regulate functions as diverse as iron uptake, Zn^{2+} transport and peroxide stress responses (Gaballa and Helmann, 1998; Chen *et al.*, 1995; Escolar *et al.*, 1999). Fur homologues are ubiquitous in Gram-negative bacteria, and are being increasingly discovered in Gram-positive species too.

The Fur protein of *E. coli* is a 17-kDa polypeptide and acts as a transcriptional repressor of iron-regulated promoters by virtue of its Fe^{2+}-dependent DNA binding activity (Bagg and Neilands, 1987b; Bagg and Neilands, 1987a). Native Fur exists as dimers, with each monomer possessing amino-terminal DNA-binding motif and carboxyl-terminal metal-binding domains (Coy and Neilands, 1991). Histidine and cysteine residues in the latter are thought to be the primary metal binding ligands. Two divalent ions are bound per monomer: a tightly-associated structural Zn^{2+} ion and a reversibly-bound Fe^{2+} ion (Althaus *et al.*, 1999). Very recently, it has been suggested that metal binding induces a conformational change resulting in a non-classical helix-turn-helix motif, which is the segment of the amino-terminal responsible for DNA-binding (de Peredo *et al.*, 2001).

Under iron-rich conditions, Fur binds its co-repressor Fe^{2+}, and acquires a configuration able to bind target DNA sequences (Fur boxes) (Bagg and Neilands, 1987b). This binding inhibits transcription from virtually all the genes and operons repressed by the metal. Conversely, when iron is scarce, the equilibrium is displaced and Fe^{2+} is released, the RNA polymerase is able to access cognate promoters, and the genes for the biosynthesis and transport of virulence genes, siderophores and other iron-related functions are expressed.

The consensus Fur box was originally thought to be a 19 bp palindrome motif (5' GATAATATAATCATTATC 3'), which together with the dimeric nature of the protein, suggested that Fur-DNA interaction was similar to that of classical bacterial repressors (where a protein dimer recognises a palindromic DNA sequence). However, this model was inconsistent with results from hydroxyl radical footprinting which revealed that Fur wrapped helically around the operator DNA rather than interacting with it on only one side of the helix, and the fact that many iron-regulated promoters appeared to have multiple, overlapping, boxes. Hence, Escolar *et al.* have suggested that the Fur binding sequences would be better interpreted as arrays of hexameric repeats (consensus 5' NATA/TAT 3') (Escolar *et al.*, 1999).

The revised view of Fur boxes as arrays of shorter sequence motifs helped explain why no individual bases appeared essential for Fur-binding in mutagenesis experiments (Stojiljkovic *et al.*, 1994). It also provides an explanation for why no natural Fur binding sites had a footprint less than 31 bp although the minimal operator was only 19 bp, and why gradual polymerisation of Fur around this

operator occurs with increasing Fur concentration. Reanalysis of the aerobactin siderophore promoter suggested that the sequences adjacent to the minimal operator were not casual but consisted of hexameric arrays able to interact with Fur (Escolar *et al.*, 2000). The higher overall binding affinity of extended binding sites such as those found in the aerobactin promoter compensate for some degree of sequence divergence. The affinity for specific promoters could vary depending on the number of repeats present and the conservation of their sequences, thus generating a hierarchy of transcriptional responses depending on small changes in cellular iron status. In other words, the combination of repetitive sequence elements that allow cooperative binding of the Fur protein in extended promoter regions explains how a relatively simple protein controls a complex regulon in a gradual fashion (Escolar *et al.*, 2000; Escolar *et al.*, 1999).

It is now known that some Fur homologues respond to metals other than Fe^{2+}. Members of the PerR sub-family are manganese-responsive (Bsat *et al.*, 1998; Chen *et al.*, 1995; Horsburgh *et al.*, 2001b), whereas their Zur counterparts bind Zn^{2+} (Gaballa and Helmann, 1998; Patzer and Hantke, 2000). Fur and/or its homologues are also known to regulate diverse functions not directly related to iron metabolism or virulence, leading to suggestions that they should be considered to be global regulatory proteins. Activities regulated include cellular processes such as the acid shock response (Hall and Foster, 1996), defence against reactive oxygen species (Chen *et al.*, 1995; Tardat and Touati, 1993) and metabolic pathways (Stojiljkovic *et al.*, 1994; Hantke, 1987). Nevertheless, Fur is principally a regulator of iron acquisition and uptake whereas the PerR and Zur regulons are mainly concerned with oxidative stress responses and zinc homeostasis, respectively. The *Y. pestis*

cluster 9 permease, which seems to transport both Mn^{2+} and Fe^{2+}, is regulated by both these cations via Fur (Bearden and Perry, 1999). As previously mentioned above, the *B. subtilis* and *E. coli* Adc-like cluster 9 zinc permeases Ycd and Znu are regulated in a zinc-dependent fashion by Zur (Patzer and Hantke, 1998; Gaballa and Helmann, 1998; Patzer and Hantke, 2000).

The DtxR Family of Metalloregulators

DtxR, first identified as the repressor of diphtheria toxin synthesis in *C. diphtheriae* (Boyd *et al.*, 1990), was the first example of a class of metal-dependent transcriptional repressor proteins found mainly in Gram-positive bacteria including *Mycobacterium tuberculosis* (designated IdeR) (Schmitt *et al.*, 1995), *Rhodococcus equi* (IdeR) (Boland and Meijer, 2000), *S. gordonii* (ScaR) (Jakubovics *et al.*, 2000) and *S. epidermidis* (SirR) (Hill *et al.*, 1998). Although the regulatory role of DtxR in iron uptake and virulence gene expression is functionally-analogous to that of Fur, the two proteins are not homologous and bind differently to DNA.

C. diphtheriae DtxR is a 25.3 kDa iron-dependent global regulatory protein governing the expression of at least seven different genes (Tao *et al.*, 1994; Holmes, 2000). These include *tox* (diphtheria toxin) and *hmuO* (haem oxygenase). Two of the genes are predicted to encode a 38.1 kDa ferric siderophore receptor (homologous to *B. subtilis* ferric siderophore receptor FhuD) and a 15 kDa transcriptional regulator homologous to AraC, respectively. The functions of the remaining three gene products have yet to be established. Interestingly, the *hmuO* gene is under the dual regulatory control of DtxR and a haem-responsive two-component signal

transduction system (Schmitt, 1999). Transcription of *hmuO* is thus repressed by holo-DtxR in high-iron conditions and activated by the response regulator ChrA in low-iron environments containing haem or haemoglobin (Schmitt, 1999).

The structures of DtxR and *M. tuberculosis* IdeR have been determined (Pohl *et al.*, 1999a; Pohl *et al.*, 1999b; Qiu *et al.*, 1996). They consist of three distinct domains: an N-terminal DNA-binding domain containing a helix-turn-helix motif, a central α-helical dimerisation domain containing the key metal ion co-ordinating residues, and a C-terminal SH3-like domain thought to be involved in metal ion binding and re-orientation of the DNA-binding domain upon metal ion activation (Pohl *et al.*, 1999b). Some homologues, e.g. TroR (*T. pallidum*) and MntR (*B. subtilis*), lack the SH3-like terminal domain (Posey *et al.*, 1999; Que and Helmann, 2000). Co^{2+}, Ni^{2+}, Mn^{2+} and (to a lesser extent) Zn^{2+} had been shown to promote binding of DtxR to its target *in vitro* (Tao *et al.*, 1992; Schmitt *et al.*, 1992), but DtxR appears to be specific for Fe^{2+} *in vivo*.

The consensus recognition sequence for *C. diphtheriae* DtxR operators consists of an interrupted 9-bp AT-rich palindrome within a 19-bp sequence termed the DtxR box, 5' TTAGGTTAGG/CCTAACCTAA 3' (Lee *et al.*, 1997). DNase I footprinting assays revealed that DtxR binding protected a region approximately 30 bp long encompassing the dyad axis of the corresponding operator (Schmitt *et al.*, 1992). *C. diphtheriae* DtxR and *M. tuberculosis* IdeR boxes that have been characterised so far overlap the -10 region (Lee *et al.*, 1997; Gold *et al.*, 2001). Appropriately, regulatory proteins that bind to σ^{70}-like promoters at regions centred downstream from position -30 almost always function as repressors. Some DtxR-specific

operators exhibit high homology with the consensus sequence in only one arm of the palindrome. The most striking example is IRP4, which is identical with the consensus sequence in the right arm but has only 3 of 9 matching nucleotides in the left arm (Lee and Holmes, 2000). The DtxR box is now known to be conserved across various Gram-positive organisms.

In 1998, the *S. gordonii* cluster 9 ABC transporter ScaCBA was shown to be a manganese permease. Because its expression could be up-regulated by limiting Mn^{2+}, it was suggested that transcription of the *scaCBA* operon was under the control of a Mn^{2+}-responsive transcriptional regulator (Kolenbrander *et al.*, 1998). Subsequently, Hill *et al.* identified immediately upstream of the *S. epidermidis* cluster 9 operon *sitCBA* a gene that encoded a DtxR-like protein, SirR (Hill *et al.*, 1998). They were also able to identify a 14 bp motif (termed the Sir box) in the promoter of *sitCBA* just downstream of the -10 element that resembled the *C. diphtheriae* consensus binding sequence and to show that SirR retarded the migration of a synthetic oligonucleotide containing the Sir box sequence in the presence of iron or manganese (Hill *et al.*, 1998). A DtxR-like protein was also discovered in *S. gordonii*, and Jakubovics *et al.* were able to demonstrate via experiments with mutants and gel mobility shift and DNase I footprinting assays that ScaR bound to the promoter and regulated the expression of the *S. gordonii* cluster 9 operon *scaCBA* in a manganese-dependent manner (Jakubovics *et al.*, 2000). The gel shift and DNase I assays demonstrated the binding of purified recombinant ScaR to the *scaCBA* promoter, and inactivation of *scaR* constitutively derepressed the *scaCBA* operon. TroR, a DtxR-like protein in *T. pallidum*, has also been shown via mobility shift and DNase I footprinting assays to be a manganese-specific

metalloregulator that represses the transcription of a putative manganese permease (Posey *et al.*, 1999). The *B. subtilis* MntR protein regulates MntH, a proton-dependent manganese transporter belonging to the NRAMP family, in a manganese-responsive manner (Que and Helmann, 2000). Unusually, MntR was also reported to positively regulate the putative ABC manganese permease *mntABCD* under low Mn^{2+} conditions, and that its binding to the *mntA* promoter was metal-independent (Que and Helmann, 2000). Recently, it has also been reported that the cluster 9 operon of *S. mutans* is also regulated by a DtxR-like protein (Spatafora *et al.*, 2001). These findings suggested a model that might be applicable to the Psa-like cluster 9 ABC transporters, i.e. those thought to belong to the subcluster proposed to be manganese permeases. Given the close homology of the *efaCBA*, *sitCBA* and *scaCBA* operons, it is likely that the *E. faecalis* cluster 9 operon *efaCBA* is a manganese permease regulated in a similar manner.

Aims and Objectives

The enterococci are now recognised as being significant pathogens, particularly in the nosocomial setting. The increasing prevalence of antibiotic-resistance has led to significant morbidity and mortality. While considerable research has been done on microbial iron-sequestration mechanisms, comparatively little attention has been paid towards other divalent transition metal ions such as manganese. A serum-induced lipoprotein expressed in *E. faecalis* isolates from patients with enterococcal endocarditis, EfaA, had previously been cloned (Lowe *et al.*, 1995). The gene encoding that lipoprotein has been found to be the third gene of a trigenic operon.

The products of this operon constitute a novel ABC transporter system (EfaCBA) in *E. faecalis*.

The *E. faecalis* cluster 9 operon *efaCBA* is highly homologous with those found in several bacteria to be manganese permeases regulated by DtxR-like proteins. These findings suggest that *efaCBA* is likely to be a manganese permease, and likely also to be regulated in a similar manner by a protein termed EfaR. The *efaCBA* operon and its transcriptional regulator are potential drug targets. As EfaA was expressed in all isolates from patients with enterococcal endocarditis, interfering with the expression of this operon could potentially prove to be a novel method of preventing or treating enterococcal endocarditis. Hence, the elucidation of the precise cellular functions and roles of these proteins in the enterococci are of interest. The primary objectives of this study were to:

- generate *E. faecalis* mutants in which either the *efaA* or the *efaR* genes had been inactivated by insertion duplication, and
- investigate the effects of divalent transition metal cations on the expression of the *efa* operon of *Enterococcus faecalis*, and
- define the substrate specificity of EfaCBA, and
- elucidate the regulatory role of EfaR with respect to EfaCBA expression and global metalloregulation, and
- analyse the interaction of EfaR with target DNA sequence and identify its cation specificity.

Chapter 2: Methods

Materials

All reagents were supplied by Sigma Chemical Co. (Poole, Dorset) unless otherwise stated. Electrophoresis-grade agarose, EDTA, ethanol, formamide, isopropanol, glucose, $MgCl_2$, and $MnCl_2$ were supplied by Merck Ltd (Poole, Dorset). Agar, Columbia Blood Agar, M17 broth, Yeast Extract, and Brain Heart Infusion broth were obtained from Oxoid (Basingstoke, Hampshire). Sterile Milli-Q water was used in all experiments except where otherwise stated.

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in **Tables 2.1** and **2.2**, respectively. *E. coli* cultures were routinely grown in Luria-Bertani (LB) medium (bacto-tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, pH 7.5) at 37°C with shaking. The medium was supplemented with ampicillin 100 μg/ml, chloramphenicol 50 μg/ml, kanamycin 50 μg/ml or tetracycline 10 μg/ml as required.

Enterococcal cultures were grown statically at 37°C in Brain Heart Infusion (BHI) broth (Oxoid) or tryptone yeast extract HEPES-buffered glucose (TYHG) medium (0.25% [w/v] Bacto-tryptone [Difco], 0.25% [w/v] yeast extract, 0.5% [w/v] glucose and 25 mM HEPES, pH 7.3). When an especially rich medium was required, cells were grown in BHI supplemented with 1% yeast extract (BHY broth). BHY medium

was supplemented with 5 to 10 μg/ml tetracycline or 1.5 to 2 mg/ml kanamycin as appropriate. To remove divalent transition metal ions from BHI or TYHG broth, Chelex-100 (Bio-Rad) treatment was used (overnight stirring with 80 g/L resin at 4°C). The resin was pre-treated according to the manufacturer's instructions to remove residual iminodiacetic acid moieties. Following Chelex treatment and sterilisation, the broth was supplemented with 1 mM $MgCl_2$. $MnCl_2$, $CuCl_2$, $FeCl_3$, $NiCl_2$ and $ZnCl_2$ were added to 10 μM as appropriate.

Table 2.1. Bacterial strains.

Strain	Description	Source/reference
E. faecalis JH2-2	Wild-type, standard laboratory strain, plasmid-free	Jacob and Hobbs (1974)
E. faecium UB1	Wild-type clinical isolate	Birmingham Heartlands Hospital, Birmingham, UK
E. coli DH5α	*endA1*, *hsdR17*, *supE44*, *thi-1*, *recA1*, *gyrA* (Nal^R), *relA1*, Δ(*lacZYA-argF*), *U169*, *deoR*$^+$	Promega
E. coli XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F'*proAB*, *lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
E. coli BL21(DE3)pLysS	F^-, *ompT*, *hsdS*$_B$ (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3), [pLysS Cam^R]	Stratagene

Abbreviations: Cam^R, chloramphenicol resistant; Nal^R, nalidixic acid resistant; Tet^R, tetracycline resistance.

Measurement of Optical Density

Growth of bacterial cultures was routinely monitored by measuring optical density at 600 nm in a Spectronic 601 spectrophotometer (Milton Roy).

Table 2.2. Plasmids.

Plasmid	Description	Source/reference
pSF143	General cloning vector, Tet^R	Tao L. (1998). (Gift from H. Jenkinson)
pFW13	Kan^R cassette plasmid	Podbielski *et al.* (1996). (Gift from H. Jenkinson)
pBluescript SK(-) or pSK(-)	General cloning vector, Amp^R	Stratagene
pGEM-T	PCR cloning vector, Amp^R	Promega
pGEM:*scaC*p	pGEM-derived, contains *S. gordonii* *scaC* promoter region	Jakubovics *et al.* (2000). (Gift from H. Jenkinson)
pGEM:*efaC*p	pGEM-derived, contains *E. faecalis* *efaC* promoter region and internal region of *efaC* for use as control.	This study
pGEM:*efmC*p	pGEM-derived, contains *E. faecium* *efmC* promoter region	This study
pGEM:*efaR*p	pGEM-derived, contains *E. faecalis* *efaR* promoter region	This study
pCal-c	Amp^R, calmodulin binding peptide (CBP)	Stratagene. (Gift from H. Jenkinson)
pCal-c:*efaR*	Derived from pCal-c, contains *efaR* ORF with CBP fused to 3' end of ORF	This study
pSK+:GP19	Derived from pBluescript SK(+), contains *efaA*	Lowe *et al.* (1995)
pSF143:*efaA*	pSF143-derived integrational vector, Tet^R, contains internal fragment of *Bam*HI/*Xba*I 0.75 kb *efaA*	This study
pSF143:*efaR*	pSF143-derived integrational vector, Tet^R, contains internal fragment of *Bam*HI/*Xba*I 0.5 kb *efaR*	This study
pKan:*efaA*	pSK(-)-derived integrational vector, Kan^R, contains internal fragment of *Bam*HI/*Xba*I 0.75 kb *efaA*	This study
pKan:*efaR*	pSK(-)-derived integrational vector, Kan^R, contains internal fragment of *Pst*I/*Bsp*120I 0.5 kb *efaR*	This study

Abbreviations: Tet^R, tetracycline resistant; Kan^R, kanamycin resistant, Amp^R, ampicillin resistant.

Identification of Bacteria

Gram Staining

Bacteria were dispersed in a drop of water on a microscope slide, which was then left to dry. The bacteria were fixed by passing the slide through a flame three times. The slide was flooded with crystal violet solution and left for 30 seconds. This solution was washed off with Lugol's iodine solution, then flooded with that solution and left for 30 seconds. Iodine/acetone was then used to wash the slide; the slide was then flooded with iodine/acetone and left for 30 seconds. Next, the slide was washed with distilled water, followed by safranin. The latter was allowed to stain for 30 seconds before being drained off. The slide was then washed with distilled water and allowed to dry.

Catalase Test

A colony of bacteria was mixed with water on a slide. A few drops of hydrogen peroxide were then added and gas evolution observed.

Identification of Enterococci

An API 20 Strep kit (bioMerieux, Basingstoke, Hants.) was used to confirm the identity of various species of enterococci. After having ascertained that the bacteria of interest were Gram-positive and catalase-negative, a well-isolated colony was suspended in 0.3 ml of water and grown overnight on Columbia Blood Agar or BHI agar. The cells were then swabbed off the plate and suspended in 2 ml of water such

that a suspension was produced that possessed a turbidity greater than 4 McFarland Units.

Each well in the first half of the test strip was inoculated with 100 µl of bacterial suspension; the second half was filled with bacterial suspension mixed with GP medium. The cupules of each well containing an anaerobic test was filled with 200 µl of mineral oil. The strips were then incubated for 4 hours at 37°C. The addition of extra reagents were necessary to reveal the results of some of the tests. One drop each of reagents VP-A and VP-B were required for the pyruvate (VP) test, two drops of ninhydrin for the Hippurate test and one drop each of Zym-A and Zym-B to the rest of the aerobic tests. After ten minutes, the colours of each of the wells was noted; these were used to obtain a 7-digit profile number which was faxed/phoned to API for identification.

Preparation, Analysis and Manipulation of DNA

Polymerase Chain Reaction

Reaction mixtures (100 µl) were prepared with 200 µM each of deoxynucleotides, 2 mM magnesium chloride, 10 µM primers and 10 µl 10x reaction buffer (all supplied by Gibco BRL, Paisley, Scotland). Template DNA (up to 10 ng plasmid DNA, or up to 500 ng genomic DNA) was added and 1 unit of Platinum *Taq* DNA polymerase (Gibco BRL) used in each reaction. Each reaction was overlaid with 50 µl mineral oil to prevent evaporation.

Amplifications were carried out in a Crocodile II thermocycler (Appligene, Watford, UK) with an initial denaturation step at 94°C for 2 minutes followed by 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C, 1 minute per 1 kb). The reaction was finished with an extended elongation step (72°C for 5 minutes). PCR products were analysed via horizontal agarose gel electrophoresis.

Primers for *E. faecalis* and *E. faecium*

Primers were prepared in lyophilised form by Gibco BRL (Paisley, Scotland). They were reconstituted and stored at -20°C or -70°C on receipt. The primers used are listed in **Table 2.3**.

Primers JCF7F and JCF7R amplified a 196 bp fragment of *efmA*, while primers YLL1F and YLL1R amplified a 544 bp fragment of *efmC*. They were used both in general PCR and to produce digoxigenin-labelled (DIG-labelled) probes by DIG-labelling PCR (DIG-PCR).

Primers YLL2F and YLL2R (with *Xba*I and *Bam*HI restriction sites built into the 5' ends, respectively) amplified a 503 bp fragment of *E. faecalis efaR*, primers YLL3F and YLL3R (*Xba*I and *Bam*HI, respectively) were used to amplify a 313 bp fragment of *E. faecalis luxS* and primers YLL4F and YLL4R (*Xba*I and *Bam*HI, respectively) amplified a 683 bp fragment of *efaA*. These primers were used to produce DNA inserts for insertion duplication and allelic replacement mutagenesis, to produce DIG-labelled probes by DIG-PCR and in general PCR.

Table 2.3. Oligonucleotides.

Primers	Sequence	Gene
JCF1F	GCGAAAGCTTCTGAAGCGGAC	405 bp fragment of *efaA*
JCF1R	GCATTTAAATCATAAGCTTTG	405 bp fragment of *efaA*
YLL1F	CGGACCAAACGGTGCAGGAAAATCA	544 bp fragment of *efmC*
YLL1R	CTGGACCGGAAGCGATCAGCTGTTT	544 bp fragment of *efmC*
YLL2F	AatgctctAgaGcTTCTGGACTCGATGTTTCGGCAGCT	503 bp fragment of *efaR*
YLL2R	aaacgggaTcccgCATTATAAATGGTGATCGGTCCTTC	503 bp fragment of *efaR*
YLL8F	CccgctCTagagcCGGGAATCAAGCCGCTGAAAAGAAA	743 bp fragment of *efaA*
YLL8R	CcGggggaTCcgGGCAAGAGAGTCTGTGAAAAGTGTA	743 bp fragment of *efaA*
YLL11F	CggggATccGTTTTCCTCACTTTCTCTAATCAACGG	*efaR*
YLL11R	cgGgCtagcAATCGCGAAGACTATTTAAAATTAATT	*efaR*
YLL14F	GgGgaAttcAAGCGCTTGTATTTAGGTGC	*efaC* promoter
YLL14R	GgGgaaTTCTTTCTTTCCTGGGCGTTTTA	*efaC* promoter
YLL16F	CgggaaTTcACATCCCTTGCAAAAGCATT	*efaR* promoter
YLL16R	GcggaattctagTCTTCGCGATTTGGTGT	*efaR* promoter
YLL17F	gggggttaacTAGTTAAACTATATTTAAATA	*efmC* promoter
YLL17R	ggcGaattccGGCTGTCCAGATAAGGTGAC	*efmC* promoter
KanF	GAGCCTTGGGAAGATGAAGTTTTTA	1044 bp fragment of *aacA*
KanR	AATTCCAGAATCTCCAAAATCAATT	1044 bp fragment of *aacA*
NSJ1F	AGCTGTTCAAGCGTTAACCG	*efaC*
NSJ1R	AATCACCTCAGCACCTTGGG	*efaC*
SP6	CGATTTAGGTGACACTATAG	Lab stock
T7	TAATACGACTCACTATAGGG	Lab stock

[a] Bases in lower case indicate mismatched bases introduced to create restriction enzyme sites (underlined).

Primers incorporating restriction sites for *Bam*HI and *Nhe*I (YLL11F and YLL11R, respectively, **Table 2.3**) were designed to amplify *efaR*; the resulting amplimer (672 bp) was ligated into the vector pGEM-T (Promega) to give pGEM:*efaR* and transformed into *E. coli* DH5α. To produce recombinant EfaR protein, the plasmid was digested and ligated into *Nhe*I- and *Bam*HI-digested pCal-c (Stratagene), generating pCal-c:*efaR*. This was transformed into *E. coli* BL21(DE3)pLysS, and transformants selected on LB agar containing 50 mg/L ampicillin.

For gel shift analyses, DNA targets in the region of 100-300 bp encompassing the promoters of *E. faecalis efaC*, *E. faecalis efaR*, *E. faecium efmC* or *S. gordonii scaC*

were used. Primers YLL14F and YLL14R (**Table 2.3**), both incorporating *Eco*RI restriction sites, were used to amplify 445 bp of *E. faecalis* DNA which was ligated into pGEM-T to produce pGEM:*efaCp*. Cleavage by *Eco*RI and *Hin*cII produced a 131 bp target containing the *efaC* promoter and a 212 bp fragment used as a control fragment. Primers YLL16F (incorporating a *Hin*cII restriction site) and YLL16R (*Eco*RI) were used to amplify a 226 bp DNA segment containing the *E. faecium efmC* promoter; the resulting plasmid was designated pGEM:*efmCp*. The *efaR* promoter was amplified using primers YLL15F and YLL15R (*Eco*RI and *Hin*cII, respectively). The 226 bp *scaCp* target DNA has been described previously (Jakubovics *et al.*, 2000). The *Eco*RI restriction sites were incorporated to facilitate end-labelling of the DNA. All sequences were checked by restriction analysis and DNA sequencing.

Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was used to resolve DNA fragments between approximately 100 bp and 10,000 bp. Electrophoresis was performed in Bio-Rad DNA subcells (Bio-Rad, Richmond, Ca.). The running buffer comprised 1x TAE or 1x TBE. Electrophoresis-grade agarose (0.7% to 1.5%) was dissolved in the appropriate running buffer at high temperature in a microwave oven. The solution was allowed to cool to approximately 60°C before being poured into a casting tray and allowed to set.

DNA samples were mixed with 6x loading buffer (10 mM Tris, 25% glycerol, 0.25% bromophenol blue, 0.5% SDS, 0.05 M EDTA) in a 5:1 ratio and loaded onto

the gels. Appropriate DNA standards were also loaded. Generally, a 1 kb ladder (Helena BioSciences) was used for DNA samples >1 kb, and a 100 bp ladder (Gibco BRL) for samples <1 kb. Electrophoresis was generally carried out at 80 V for one and a half hours, after which the gel was soaked in an ethidium bromide (0.5 μg/ml) solution for half an hour. The stained gel was then visualised on a long wave (360 nm) UV transilluminator and photographed where appropriate.

Extraction of DNA from Agarose Gels

DNA was recovered from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK), in accordance with the manufacturer's instructions.

Extraction of Chromosomal DNA

Large Scale Chromosome Extraction

Large scale chromosome extraction was carried out using a protocol adapted from the methods of Skjold *et al.* (Skjold *et al.*, 1987). An overnight culture (50 ml) of *E. faecalis* in BHI broth was centrifuged at 4,000 x *g* for 10 minutes. The pellet was washed twice in 20 ml ice-cold 0.2 M sodium acetate (pH 6.0). The solution was then centrifuged at 4,000 x *g* for 10 minutes and resuspended in 5 ml TE glucose (25% [w/v] glucose, 100 mM Tris·Cl, 10 mM EDTA, pH 7.0).

The suspension was incubated with mutanolysin 20 μg/ml for 2 hours at 37°C. Following centrifugation at 4,000 x *g* for 15 minutes, the pellet was suspended in 4.5 ml TE. 0.5 ml of 10% (w/v) N-laurylsarcosine was added and the tube inverted

gently to mix. The reaction was incubated at 37°C for 30 minutes. Pronase (self-digested, 37°C, 1 hour) was added to 500 μg/ml and the reaction was incubated for 1 hour at 37°C. The solution was then extracted with phenol-chloroform. DNA was precipitated by adding 0.6 to 0.7 volume of room-temperature isopropanol. The DNA was recovered by spooling the thread-like precipitate onto a sterile pasteur pipette and transferred to a fresh microfuge tube. It was washed by rinsing with 70% ethanol, dried under vacuum and suspended in 100 μl of TE buffer or water.

Small Scale Chromosome Extraction

Small-scale chromosome extraction was carried out using the Igi Genie Genomic kit from Helena Biosciences (Sunderland, UK), in accordance with the manufacturer's instructions.

Preparation of Plasmid DNA

Small Scale Plasmid Extraction

Small scale plasmid extraction was performed using the Wizard *Plus* SV Mini Prep kit (Promega, Madison, WI), in accordance with the manufacturer's instructions.

Large Scale Plasmid Extraction

Large scale plasmid extraction was performed using the Qiagen Plasmid Maxi Kit (Qiagen), in accordance with the manufacturer's instructions.

Restriction of Plasmid DNA

DNA restrictions were carried out using enzymes purchased from New England Biolabs (Beverly, MA) or Helena BioSciences (Sunderland, UK). The sample, enzyme (5 to 20 U/μg plasmid DNA) and appropriate buffer were added to a final volume of either 20 μl or 50 μl as appropriate. Reaction mixtures were incubated at 37°C for 1 to 4 hours. Reactions were stopped by the addition of 12.5 mM EDTA (pH 8.0) or by heating the solution to 65°C for 20 minutes (80°C for *Bam*HI). Digests were analysed by agarose gel electrophoresis.

Dephosphorylation of Plasmid DNA

Treatment with calf intestinal alkaline phosphatase (CIAP) was used to remove the 5'-phosphate groups from blunt-ended linear cloning vectors prior to ligation to prevent vector self-ligation. CIAP (0.01 unit per pmol ends, Gibco), CIAP reaction buffer (10x, Gibco) and water were added to pre-digested vector to a final volume of 100 μl. The reaction mixture was incubated at 37°C for 1 hour and stopped by addition of 2 μl of 0.5 M EDTA and incubation at 65°C for 20 minutes. Dephosphorylated DNA was purified by phenol-chloroform extraction and ethanol precipitation.

Ligation of Plasmid DNA

Ligation of vector and foreign DNA was carried out as described by Sambrook *et al.* (Sambrook *et al.*, 1989). Reactions were set up by transferring 50 ng of vector DNA to a microcentrifuge tube. An equimolar amount of insert DNA was added. The following equation was used to determine the required amount of insert DNA:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

The mixture was made up to a final volume of 10 µl by addition of 1 µl DNA ligase buffer (10x), 1 µl of 1 U/µl T4 DNA ligase (Helena BioSciences) and water. The reaction was incubated at 16°C for 1 to 16 hours; 1 to 2 µl of each ligation reaction used to transform *E. coli* cells.

The pGEM-T Vector System (Promega), comprising the plasmid pGEM-T and associated reagents, was used in the cloning of certain PCR products. This plasmid is provided in a linearised form with a single thymidine overhang at both its 3'-ends. The presence of these overhangs greatly improves the efficiency of ligation of PCR products – which possess complementary 3'-adenine overhangs - into these plasmids. Ligation reactions were carried out as instructed by the manufacturer.

Purification of DNA

Purification by Ethanol Precipitation

Two methods of ethanol precipitation were used to clean-up and/or concentrate DNA. The first uses ammonium acetate; the second, lithium chloride. For most DNA samples, 0.5 volumes of ammonium acetate (7.5 M) and 2.5 volumes of 95% ethanol were added to 1 volume of sample. The resulting mixture was centrifuged at 14,000 x *g* for 15 minutes, following which the supernatant was discarded. The pellet was then rinsed in 250 µl of 70% ethanol and centrifuged at 14,000 x *g* for 5 minutes. The ethanol was removed, the pellet air-dried and the DNA suspended in 10-25 µl of TE buffer or water.

With DIG-labelled samples, when necessary, nucleic acids were precipitated by mixing 0.1 volume of 4 M LiCl and 2.5 to 3 volumes of chilled ethanol with 1 volume of sample. The mixture was incubated at -70°C for 30 minutes, after which it was centrifuged at 13,000 x *g* for 15 minutes. The ethanol was decanted, the pellet washed with 100 µl of ice-cold 70% ethanol and centrifuged at 13,000 x *g* for 5 minutes. The pellet was dried and resuspended in 50 µl of TE buffer or water.

Purification using the QIAquick PCR Purification Kit

DNA was cleaned up following PCR, and also after restriction, ligation and random hexamer labelling reactions using the QIAquick PCR purification kit (Qiagen), in accordance with the manufacturer's instructions.

Purification by Phenol-Chloroform Extraction

Equal volumes of DNA-containing solution and phenol-chloroform-isoamyl alcohol (25:24:1, saturated with 50 mM Tris-HCl , pH 8.0) were mixed by vortexing, and then separated by centrifugation at 14,000 x *g* for 5 minutes. The aqueous phase was transferred to a fresh tube and the process repeated until no precipitate formed at the interface. The solution was then extracted once with chloroform:isoamyl alcohol (24:1).

Estimation of DNA Concentration by Ethidium Bromide Dot Quantification

The following DNA standard solutions were prepared in TE buffer (pH 8.0): 0 μg/ml, 1 μg/ml, 2.5 μg/ml, 5 μg/ml, 7.5 μg/ml, 10 μg/ml, 20 μg/ml. DNA was obtained from the pBR322 plasmid (Sigma). Each standard DNA solution (4 μl) was mixed with 4 μl ethidium bromide 1 μg/ml. The unknown DNA solutions were prepared in the same way. A piece of Saran wrap was placed on a UV transilluminator and the standard and the unknown DNA solutions were spotted side by side on the plastic wrap. The fluorescence of the standard and unknown DNA solutions was compared under UV light to estimate the DNA concentration of the unknown solutions.

Spectrophotometric Determination of Nucleic Acid Concentration

Quantitation of DNA and RNA was performed using a GeneQuant II spectrophotometer (Pharmacia Biotech, St. Albans, Herts.). Nucleic acid concentration was determined from absorbance at 260 nm; the ratio of absorbances at 260 nm and 280 nm was used as an indicator of nucleic acid purity.

Sequencing and Computer-Assisted Sequence Analysis

Sequencing of the plasmids constructed in this study were carried out at the automated DNA sequencing facility in the Department of Biology and Biochemistry at the University of Bath. Sequence analyses were performed using the Wisconsin Genetics Computer Group (GCG) software package on the University of Bath GNOME Unix server. The *E. faecalis* DtxR-like protein was identified by BLAST searching the unannotated sequence of the *E. faecalis* V583 chromosome at the TIGR website (http://www.tigr.org). The BLAST search facilities of the National Library of Medicine, Washington, DC (NCBI) were used to search for homologues.

Nucleotide accession numbers

The sequences of the *efaR* gene of *E. faecalis* JH2-2 was submitted to the DDBJ/EMBL/GenBank databases under Accession No. AF409093.

Transformation of Bacterial Cells

Heat Shock

Preparation of Competent *E. coli* for Heat Shock

A single fresh colony of *E. coli* DH5α was inoculated into 5 ml LB broth and grown overnight at 37°C. This primary culture was diluted 1:100 and grown until the OD_{470} reached 0.48. The suspension was chilled on ice for 5 minutes, then centrifuged at 4,000 x *g* for 5 minutes. The cells were suspended in $2/5^{th}$ volume transforming buffer I (30 mM KCl, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v/v glycerol, pH 5.8). After chilling on ice for 5 minutes, the cells were centrifuged at 4,000 x *g* for 5 minutes at 4°C. The cells were then suspended in $1/25^{th}$ volume of transforming buffer II (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% v/v glycerol pH 6.5). They were left on ice for 15 minutes. The cell suspension was divided into 200 µl aliquots and snap frozen on dry ice/ethanol and stored at -70°C.

Transformation of *E. coli* by Heat Shock

Competent cells were thawed at room temperature. After chilling on ice for 10 minutes, the DNA was added and the suspension was mixed gently. The suspensions were left on ice for 30 to 45 minutes. Heat shock was carried out at 37°C for 2 minutes. Immediately afterwards, the cells were cooled on ice for 2 minutes. Four volumes of LB broth were added and the suspensions were incubated at 37°C for 1 hour. The cells were pelleted and suspended in 100 µl LB broth. All of the mixture was plated out onto LB agar plates containing appropriate antibiotic.

Blue/White Selection

Where blue/white selection was to be used, 40 µl of X-Gal (20 mg/ml in dimethylformamide) and 4 µl of 0.84 M IPTG were spread on the agar surface of each plate. The plates were dried at 37°C for four hours. Bacterial colonies containing the recombinant plasmid appeared white, whilst those having the intact *lacZ* gene appeared blue, enabling easy selection of recombinants.

Electroporation

Preparation of *E. faecalis* for Electroporation

E. faecalis JH2-2 was electroporated using a protocol adapted from the method of Cruz-Rodz and Gilmore (Cruz-Rodz and Gilmore, 1990). *E. faecalis* was inoculated into 10 ml M17-glucose broth (M17 broth with 5% glucose, pH 6.8-7.0) and incubated overnight at 37°C without aeration. After 20 hours, 1 ml was subcultured into 100 ml SGM17-glucose medium (M17-glucose with 0.5 M sucrose and 5% glycine, pH 6.8-7.0). Cells were grown for 21 hours at 37°C without aeration. Cells were harvested by centrifugation at 1000 x *g* for 15 minutes at 4°C and washed twice with 100 ml ice-cold electroporation buffer (EPB; 0.5 M sucrose, 10% glucose, 10% glycerol, pH 7.0) by resuspension followed by centrifugation. Finally, the cells were suspended in 1 ml ice-cold EPB and stored frozen at -80°C as 50 µl aliquots.

Transformation of *E. faecalis* by Electroporation

The cell suspension was thawed on ice. The DNA was mixed with 50 µl of the cell suspension, and the mixture then transferred into an ice-cold electrode-gap cuvette (Bio-Rad). Cells were electroporated at 25 µF, 200 Ω and 2.5 kV in a Bio-Rad Gene Pulser apparatus. Immediately after electroporation, 960 µl of pre-warmed SGM17-glucose (pH 6.8-7.0) with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ was added, the cells resuspended and transferred to a microfuge tube. The cells were incubated for at least 2 hours at 37°C, without aeration. Serial dilutions were performed to 10^2 dilution factor and portions (0.1 ml) were plated onto selective BHY agar. Serial dilutions up to a dilution factor of 10^5 were plated onto non-selective BHY agar to estimate the total viable count. Plates were incubated aerobically at 37°C for up to 72 hours.

Nucleic Acid Blotting Techniques and Hybridisation Analysis

Production of Digoxigenin-Labelled Probes via PCR

Digoxigenin-labelled probes for use in colony blots, Northern and Southern hybridisations were generated via DIG-labelling PCR, with reagents from Roche Applied Science (East Sussex, UK). Reaction mixtures (100 µl) were prepared with 5 mM magnesium chloride, 10 µM primers, 10 µl 10x reaction buffer, 10 µl template DNA and 0.5 µl Taq polymerase. Nucleotides dATP, dCTP and dGTP

were added to a final concentration of 200 μM, along with 180 μM dTTP and 20 μM digoxigenin-11-dUTP.

Random Primed DNA Labelling

Template DNA (1 μg) was diluted to 15 μl and denatured by heating at 95°C for 10 minutes. The DNA was chilled on ice; still on ice, 2 μl of hexanucleotide mixture (10x) and 2 μl dNTP labelling mixture (10x) and 1 μl of labelling-grade Klenow enzyme were added. The reaction was incubated overnight at 37°C. Labelling efficiency was verified prior to hybridisation.

Estimation of Probe Labelling Yield

DNA probes labelled with digoxigenin were produced by PCR for use in probing Northern membranes. Estimation of digoxigenin-labelling yield was carried out in a spot test by comparison of the DIG-labelled sample with a DIG-labelled control (Roche Applied Science). Serial dilutions were carried out to a dilution factor of 10^5 on both sample and control DNA. 1 μl of each dilution was spotted onto a piece of nylon membrane (Roche Applied Science). The membrane was baked for 30 minutes at 120°C. Chemiluminescent detection was then carried out. Spot intensities of control and sample dilutions were compared to estimate the concentration of probe.

Hybridisation with Digoxigenin-Labelled Probe

Membranes to be probed were placed in a bag and soaked for at least 1 hour at 45°C in a prehybridisation solution. The prehybridisation solution consisted of high SDS buffer (7% SDS, 50 mM sodium phosphate, 2% blocking reagent [Roche Applied Science], 5x SSC, 0.1% N-laurylsarcosine, 50% formamide). Digoxigenin-labelled probe was made up to 50 µl with water and denatured by heating at 95°C for 10 minutes. It was diluted to 25 ng/ml with an appropriate amount of high SDS buffer to produce the hybridisation solution. The prehybridisation solution in the bag was discarded and replaced with the hybridisation solution, and the membranes incubated overnight at 45°C. Following hybridisation, the membranes were washed twice in 2x SSC, 0.1% SDS for 5 minutes at 55°C, followed by two washes in 0.1x SSC, 0.1% SDS for 5 minutes, also at 55°C.

Detection of DIG-Labelled Probes

All the subsequent steps were performed at room temperature unless otherwise stated. First, the membranes were washed in Washing Buffer (0.3% Tween-20 in Buffer 1 [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) for 5 minutes, then blocked in Buffer 2 (10% Blocking Reagent in Buffer 1) for 30 minutes. Anti-digoxigenin-AP antibody (Roche Applied Science) was diluted 1:10,000 in Buffer 2 and incubated with the membrane for half an hour. The membranes then underwent three ten minute washes in Washing Buffer. These steps were carried out on a shaker. The membranes were then equilibrated in Buffer 3 (100 mM Tris·Cl, pH 9.5, 100 mM NaCl) for 2 to 5 minutes. The lumigen (CSPD [Roche Applied Science]) was diluted 1:100 in Buffer 3 and spread evenly over the membrane. This was left for 5 minutes

and then incubated at 37°C for 30 minutes. Kodak X-Omat AR film was exposed to the membrane for 1 hour and developed according to the manufacturer's instructions.

Stripping Membranes for Reprobing

Nylon membranes from Southern and colony hybridisations were stripped and reprobed as necessary. The membrane was washed in water for 1 minute. It was then incubated twice for 10 minutes at 37°C in pre-warmed alkaline probe-stripping solution (0.2 M NaOH, 0.1% SDS). Finally, the membrane was rinsed thoroughly in 2x SSC. After stripping, the membrane was either used immediately or stored in 2x SSC.

Colony Blotting Gram-Positive Bacteria

Colonies on agar plates were cooled for approximately 30 minutes at 4°C. A nylon membrane disc (Roche Applied Science) was carefully placed on the agar surface and left for approximately 1 minute, then removed and blotted briefly (colony side up) on dry Whatman 3MM paper. It was then transferred onto Whatman 3MM paper soaked with denaturation solution (1.5 M NaCl, 0.5 M NaOH). After 5 minutes, the membrane was transferred onto 3MM paper soaked with neutralisation solution (1 M Tris·Cl, 1.5 M NaCl, pH 7.5) and left for 15 minutes. Finally, it was blotted on paper soaked with 20x SET buffer (3 M NaCl, 20 mM EDTA, 0.4 M Tris·Cl, pH 7.8) for 10 minutes. The transferred DNA was fixed by baking for 30 minutes at 80°C.

Proteinase K treatment was then carried out to digest proteins which might interfere with subsequent hybridisation. The membrane was placed on clean aluminium foil and 0.5 ml of 2 mg/ml Proteinase K pipetted onto the membrane. The membrane was incubated for 1 hour at 37°C. The membrane was placed between two sheets of 3MM paper wetted with water and pressure was applied with a large bottle. This enabled cellular debris, stuck to the upper sheet of filter paper, to be removed by gently pulling off that sheet. The membrane was then hybridised with DIG-labelled probes.

Southern Blotting

Southern blotting was used to demonstrate that insert-bearing plasmid derivatives had successfully disrupted the *efaA* and *efaR* genes of *E. faecalis* JH2-2. Two types of probes were used: DIG-labelled PCR probes identical to the plasmid inserts or resistance determinants, and DIG-labelled probes based on pSF143 sequence produced via random primed DNA labelling.

Chromosomal DNA (approximately 0.5 μg) was extracted and restricted (10 to 20 units of enzyme per μg DNA, 16 hours at 37°C). The DNA digest was run overnight (12 to 16 hours) at 40 V on a 1% agarose gel, using TAE as running buffer. The gel was stained with ethidium bromide to visualise DNA fragments and to confirm subsequent transfer to the membrane.

All the following steps were performed at room temperature. First, to depurinate the DNA, the gel was soaked in 0.25 M HCl for 10 minutes, with shaking. It was then rinsed with Milli-Q water before being soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes. The gel was rinsed again in water and soaked for a further 15 minutes in denaturation solution. After further rinsing in water, the gel was soaked twice (15 minutes each time) in neutralisation solution (3 M NaCl, 0.5 M Tris-HCl , pH 7.5).

The DNA was transferred from the gel to a nylon membrane (Roche Applied Science) by upwards capillary transfer. A container was partly filled with 20x SSC, and a sheet of glass placed on the container to serve as the platform on which the transfer stack was to be built. A piece of Whatman 3MM paper was wrapped around the glass sheet to serve as a wick. The gel was placed on the blotting paper surface side down. The membrane was wetted in 20x SSC and placed on the surface of the gel. Air bubbles were removed by rolling a glass pipette over the membrane. Three pieces of Whatman 3MM paper were cut to a size slightly smaller than the membrane, wetted in 20x SSC and placed on top of the membrane. Air bubbles were removed as before. Paper towels were cut to a similar size as the Whatman paper and stacked dry on top to a height of approximately two inches. A glass plate was placed on the stack, a 0.5 kg weight added on top, and the stack left overnight (for a minimum of 16 hours) to allow capillary transfer. After transblotting, the membrane was baked for 30 minutes at 120°C to fix the DNA. Transfer efficiency was examined on a UV transilluminator. Prehybridisation and DIG-probe hybridisation were then carried out.

RNA Isolation and Northern Blotting

Overnight cultures were inoculated 1:100 into fresh medium and RNA was extracted when they reached an OD_{600} of about 0.6. Cells were harvested by centrifugation at 3310 x *g* for 10 minutes, washed once in 1 ml distilled water and resuspended in 200 μl spheroplasting buffer (26% [w/v] raffinose, 10 mM $MgCl_2$, 0.5 mM PMSF, 20 mM Tris-HCl, pH 6.8). Mutanolysin (10 µl of 10,000 units/ml) was added and suspensions were incubated at 37°C for 10 minutes. Total RNA was extracted from mutanolysin-treated cells using an RNeasy kit (Qiagen) and quantified at 260 nm.

Various precautions were taken to prevent RNase contamination. Sterile disposable plasticware was used wherever possible. Electrophoresis equipment and the laboratory accessories used in the hybridisation and washing processes were wiped with RNase-away and all solutions were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC).

RNA was fractionated at 80 V on 0.8% horizontal agarose gels containing 3% (v/v) formaldehyde. Prior to loading, 10 µl of RNA (≈10 µg) was mixed with 30 µl of sample buffer (7 µl formaldehyde, 4 µl 20x MOPS, 2 µl ethidium bromide, 20 µl formamide). The mixture was heated at 90°C for 15 minutes and quenched on ice to denature the RNA. Bromophenol blue (2.5 µl of a 0.4% solution) was added, vortexed and centrifuged at 14,000 x *g* for 1 minute. The RNA was then loaded onto the gel. One well was loaded with RNA marker (Sigma).

After electrophoresis for approximately two hours, the gel was soaked in water for 30 minutes to remove the formaldehyde. RNA was transferred by upwards capillary transfer as described for Southern blotting to a nylon (Roche Applied Science) or Hybond N+ membrane (Amersham Pharmacia Biotech), depending on whether the probes to be used were DIG- or radioactively-labelled, respectively. After transblotting, the membrane was baked for 30 minutes at 120°C.

DIG-labelled probes were produced and used as described above. ^{32}P-labelled probes were produced using a Prime-a-Gene kit (Promega) according to the recommendations of the manufacturer and purified on a NICK column (Amersham Pharmacia Biotech). Hybridisation was performed in Church and Gilbert medium (Church and Gilbert, 1984) at 68°C overnight. Non-specifically bound probe was removed by washing twice for 5 minutes at room temperature in 2x SSC, 0.1% SDS and twice for 20 minutes in the same medium at 68°C. The membranes were air-dried and exposed to Fuji HR-E30 X-ray film for 1 to 5 days.

Protein Manipulation and Analysis

Protein Isolation from Enterococci

Enterococcal surface proteins were extracted by grinding cells with glass beads. An overnight *E. faecalis* culture was used to inoculate pre-warmed medium. The cells were grown to late log phase and harvested by centrifugation at 8000 x *g* for 10 minutes. The cells were washed with water, resuspended in TE buffer containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and digested with lysozyme

(0.2 mg/ml) for 10 minutes at 37°C. This suspension was transferred to a 1.5 ml microfuge tube and vortexed with 0.2 g ≤106 μm glass beads (Sigma) for 1 minute, after which it was cooled on ice for 1 minute. The vortexing and cooling episodes were repeated twice. After the beads had settled, the suspension was transferred to a fresh microfuge tube and spun at 8,000 x *g* for 1 minute to pellet unbroken cells; the supernatant was recovered and centrifuged at 14,000 x *g* for 30 minutes at 4°C to pellet the envelope fragments.

Protein Separation by SDS-PAGE

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Mini Protean II system. Denaturing 4% (w/v) stacking and 10% (w/v) resolving polyacrylamide gels were used. Samples were diluted with an equal volume of 2x Laemmli sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue) (Laemmli, 1970) and then heated at 100°C for 5 minutes before loading. Electrophoresis was carried out at 200 V in running buffer (25 mM Tris, 0.1% [w/v] SDS, 192 mM glycine) until the tracking dye reached the bottom of the gel.

Reagent	10% Resolving Gel	4% Stacking Gel
40% (w/v) acrylamide:bisacrylamide 37.5:1	3.75 ml	0.9 ml
Water	5.43 ml	6.84 ml
1 M Tris-HCl , pH 8.8	5.6 ml	-
1 M Tris-HCl , pH 6.8	-	1.1 ml
10% (w/v) SDS	0.15 ml	0.09 ml
10% APS	50 μl	50 μl
TEMED	20 μl	20 μl

After electrophoresis, the gels were soaked in staining solution (0.1% [w/v] Coomassie Brilliant Blue R-250, 50% v/v methanol, 10% v/v glacial acetic acid) for 1 to 4 hours. They were then washed in destaining solution (50% v/v methanol, 10% v/v glacial acetic acid) until distinct bands could be observed.

Preparation of Dialysis Tubing

Dialysis was used to change the salt composition of protein-containing solutions. About 10 to 15 cm of Visking dialysis tubing (MWCO 10,000; Merck) was boiled for 10 minutes in a solution containing 2% (w/v) sodium bicarbonate (NaH_2CO_3) and 1 mM EDTA (pH 8.0). The membrane was then rinsed several times in distilled water and boiled for 10 minutes in 1 mM EDTA (pH 8.0). The membrane was again rinsed several times in distilled water. Pre-treated membranes not used immediately were stored in 20% ethanol at 4°C to prevent growth of cellulolytic microorganisms; they were rinsed thoroughly with distilled water prior to use.

Western Blotting

Proteins were resolved by SDS-PAGE and transferred onto Polyscreen PVDF Transfer membranes (NEN Life Science Products, Cambridge, UK) using a Mini Trans-Blot cell (Bio-Rad). Transfer was carried out at 100 V for 1 hour in transblot buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). The electroblotted proteins were detected by staining the membranes with Ponceau S (Sigma).

The membranes were blocked by incubation in 4% (w/v) non-fat dry milk powder in TBS (0.9% [w/v] NaCl, 10 mM Tris, pH 7.4) with gentle agitation at room temperature. They were then probed overnight at 4°C with monospecific polyclonal rabbit antibodies to EfaA. The antiserum was raised in New Zealand White rabbits against EfaA protein. The protein had been expressed from pSK+:GP19 in *E. coli* XL1-Blue, separated by SDS-PAGE and electroeluted from the gel. Prior to use, the antiserum was diluted 1:50 in TBS.

Blots were rinsed in TBS and secondary detection carried out using a 1:5,000 dilution of antirabbit horseradish peroxidase conjugates for 1 hour. The blots were visualised using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Life Science).

Purification of Recombinant EfaR

LB broth (0.6 L) was inoculated 1:50 from an overnight culture of *E. coli* BL21(DE3) cells harbouring pLysS and pCal-c:*efaR*. The culture was shaken at 200 rpm at 37°C until an OD_{600} of 0.5 to 0.6, at which time IPTG was added to 1 mM. The cultures were incubated for a further 3 ½ hours. Cells were collected by centrifugation and suspended in 15 ml $CaCl_2$-binding buffer (150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 mM PMSF, 50 mM Tris-HCl, pH 8.0). Lysozyme (0.2 mg/ml), RNase A (10 μg/ml) and DNase I (5 μg/ml) were added and the suspension incubated with

shaking at 4°C for 15 minutes. The suspension was then subjected to five 30-second episodes of sonication with a microtip sonicator; the sample was cooled on ice for three minutes following each sonication episode. Insoluble material was removed by centrifugation at 10,000 x *g*, 4°C for 15 minutes.

Calmodulin-affinity resin (Stratagene) was used to purify the recombinant EfaR-calmodulin-binding peptide (EfaR-CBP) from the supernatant by a batch-binding method in accordance with the manufacturer's instructions. EfaR-CBP was eluted from the resin (5 ml) in 15 resin volumes of elution buffer (0.3 M NaCl, 2 mM EGTA, 0.1 mM PMSF, 50 mM Tris-HCl, pH 8.0) and dialysed into thrombin cleavage buffer (300 mM NaCl, 2.5 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.4). The CBP tag was cleaved from 1 mg of the fusion protein by incubation with 10 μg thrombin for 16 hours. The reaction was stopped by addition of PMSF to 0.5 mM. Cleaved CBP was removed by adjusting the NaCl concentration to 200 mM, adding 1 ml calmodulin-affinity resin, incubating at 4°C for 30 minutes and then removing the resin by centrifugation.

Preparation of ^{32}P-Labelled DNA Targets for Protein Binding

Target DNA fragments for electrophoretic mobility shift assays (EMSAs) and DNase I protection assays were excised from the plasmids into which they had been cloned using *Eco*RI and *Hin*cII and extracted from a 1.5% (w/v) agarose gel using a QIAquick Gel Extraction Kit (Qiagen). The fragments were labelled with [α^{32}P]-dATP (6000 Ci/mmol, Amersham Pharmacia Biotech) using the Klenow fragment

of DNA polymerase (Helena BioSciences). Approximately 0.5 to 1 μg of DNA, 10 μCi of [α^{32}P]-dATP, 1 unit of Klenow Fragment, 4 μl of 10x Klenow buffer and unlabelled dNTPs (4 mM) were combined in a reaction volume of 40 μl. The reaction was incubated for 15 minutes at room temperature, whereupon the reaction was stopped by heating at 70°C for 5 minutes. Unincorporated nucleotides were removed using a Microcon YM-100 column (Millipore), following the manufacturer's instructions. The concentrated ^{32}P-labelled DNA was diluted to 10,000 cpm/μl.

Electrophoretic Mobility Shift Assays

Target DNA fragments were excised from the plasmids and labelled with ^{32}P as described above. Protein-DNA binding reactions (20 μl) were contained 5 mM $MgCl_2$, 40 mM KCl, 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 5% (v/v) glycerol, 5 μg BSA, 1 μg sonicated herring sperm DNA, 20,000 cpm target DNA and purified protein. In certain reactions, freshly prepared divalent metal salts such as $MnCl_2$ or $FeCl_2$ were added to 125 μM. After incubation at 30°C for 15 minutes, 10 μl of each reaction was loaded onto a 5.5% non-denaturing polyacrylamide gel, containing 2.5% glycerol (1.5 ml 10x Bis-Tris Borate Buffer 0.75 ml 50% glycerol, 2.06 ml 40% acrylamide:bisacrylamide 37.5:1, 10.69 ml water, 10 μl TEMED, 75 μl 10% [w/v] ammonium persulfate). Electrophoresis was carried out at 80 V for 1 to 1.5 hrs in Bis-Tris borate buffer (40 mM boric acid, 40 mM Bis-Tris, pH 7.6). The gels were analysed by autoradiography.

DNase I Footprint Analysis

Target DNA for DNase I protection analyses was prepared as for EMSAs. Assays were performed in a 19 μl reaction volume containing 5 mM $MgCl_2$, 40 mM KCl, 20 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$, 1 mM DTT, 5% (v/v) glycerol, 5 μg BSA, 1 μg sonicated herring sperm DNA, 100 μM $MnCl_2$, 20,000 cpm target DNA and purified protein. After incubation at 30°C for 15 minutes, 1 μl of DNase I (0.1 unit) was added. The incubation was continued for a further 1 minute, then stopped by addition of 20 μl stop solution (5 M ammonium acetate, 50 mM EDTA, 10 mg/ml yeast tRNA). Cold absolute ethanol (120 μl) was added and the mixture was chilled at -70°C for 30 minutes. Precipitated DNA fragments were harvested by centrifugation at 12,000 x *g*, 4°C for 20 minutes, washed in 0.2 ml of cold 70% (v/v) ethanol, centrifuged at 12,000 x *g*, 4°C for 10 minutes and dried. Fragments were dissolved in 6 μl of formamide/tracking dye solution, denatured at 95°C for 3 minutes and cooled rapidly on ice. The DNA fragments were separated on a denaturing 6% (w/v) polyacrylamide gel (40% [w/v] acrylamide:bisacylamide 19:1, National Diagnostics) containing 6 M Urea. Electrophoresis was carried out at 1900 V for 1 1/2 to 2 hours in 0.6x TBE. The gel was pre-run for 1 hour prior to use. Once run, the gel was dried and analysed by autoradiography using Kodak X-Omat AR film. Sizes of the fragments were estimated by comparison with a Maxam and Gilbert A+G sequencing ladder (Short Protocols in Molecular Biology, 1999).

Preparation of Maxam and Gilbert A+G Sequencing Ladders

To produce A+G ladders for use in DNase I footprinting, 20,000 cpm of ^{32}P-labelled target DNA (made up to 6 µl) was mixed with 4 µl of sonicated herring sperm DNA

(Promega) and 25 µl of concentrated formic acid. This mixture was incubated at 25°C for 5 minutes. To stop the reaction, 200 µl of hydrazine stop buffer (25 µg/ml tRNA, 0.1 M EDTA, 0.3 M sodium acetate, pH 7.0) and 200 µl chilled absolute were added and the mixture chilled in a dry ice/ethanol bath for 5 minutes. DNA was pelleted by centrifugation for 5 minutes at 15,000 x *g*. The pellet was rinsed twice with 70% ethanol. It was redissolved in 70 µl of 10% piperidine and incubated at 90°C for 30 minutes. The piperidine solution was then removed by drying under vacuum. To ensure complete removal of piperidine, the DNA was redissolved in 30 µl of water, transferred to a new tube, and the liquid removed by vacuum evaporation. The process was repeated with 20 µl of water. The DNA was finally reconstituted in 6 µl of formamide/tracking dye solution.

Chapter 3: Inactivation of Enterococcal Genes

Insertional Mutagenesis of *E. faecalis* JH2-2

The inactivation of specific genes can be a powerful tool to investigate the phenotypic traits controlled by those loci. Successful gene inactivation can often enable physiological traits to be assigned to or correlated with particular genes. For example, comparison of the pathogenicity of null mutants with that of wild-type strains can implicate or rule out a role for a particular gene in pathogenesis. Hence, attempts were made to disrupt the functioning of EfaR (this protein is discussed in greater detail in **Chapters 4** and **5**) and EfaCBA by gene inactivation in the hope of elucidating their potential roles in the virulence of *E. faecalis*.

A wide variety of mutagenesis strategies exist, ranging from random mutagenesis by chemical means to site-directed DNA-based techniques based on homologous recombination. Random mutagenesis has the considerable drawback of necessitating the screening of a large number of clones for the desired mutation with little guarantee of success. Mutagenesis by homologous recombination, being site-directed, offers a means of circumventing such problems.

Homologous Recombination

It has been known for some time that long homologous sequences of DNA can be recombined in a sequence-specific manner *in vivo*. Homologous recombination is a

mechanism utilised by the cell in events such as DNA repair and chromosomal replication. The phenomenon of homologous recombination has been most extensively studied in *E. coli* (Kogoma, 1997; Kowalczykowski *et al.*, 1994).

It is possible to manipulate the process of homologous recombination to achieve scientific ends, i.e. the inactivation of specific genes. By coupling a genetic marker (usually encoding antibiotic resistance) to a DNA sequence homologous to the target gene, it is possible to introduce that marker into a bacterial cell and ultimately to have it insert into the target gene, thereby disrupting that gene ("insertional inactivation"). In order to perform insertional inactivation by homologous recombination, the marker DNA and target gene sequence are usually delivered into the host cell by means of a shuttle vector. Shuttle vectors are plasmids normally designed to exist in more than one species, e.g. *E. coli* and *Enterococcus* spp., and usually contain an origin of replication for each of the species that they inhabit. In some cases, however, they will contain an origin of replication for one host only, with no origin of replication suitable for the target host species. For such a vector to survive in that target host, it must combine with the host chromosome and hence, such vectors are often termed "suicide" vectors.

Typically, one or two segments of the gene of interest - the "target sequence" - are cloned into the integration vector. Depending on whether one or two DNA target sequences were cloned into the vector, one of two forms of gene disruption (insertional inactivation) will occur. If only one piece of target sequence is cloned into the plasmid, recombination of the plasmid target sequence and homologous DNA on the host chromosome causes the entire plasmid to become integrated into

the chromosome, resulting in insertion-duplication of the target site. Hence, this is termed "insertion duplication", "single crossover" or "Campbell-like" insertion. A suicide vector is usually used to encourage plasmid integration into the genome. One problem that can arise with this form of insertional inactivation is structural instability of the locus, because the integrated plasmid is flanked by repeated sequences. Further recombination can occur, resulting in plasmid excision and reversion to parental chromosomal structure. The alternative form of insertional inactivation occurs if the marker is flanked on either side by target sequence, e.g. if an antibiotic marker were cloned into the middle of the target sequence, of if the marker replaced an internal portion of the target sequence. Recombination events would then occur on either side of the marker, resulting in its insertion into or its exchange with a portion of the homologous chromosomal target. Hence, this form of insertional inactivation is frequently termed a "double crossover" event or "allelic replacement". The two forms of insertional inactivation are illustrated in **Fig. 3.1**.

In theory, only bacteria that have successfully undergone recombination following transformation should express the relevant antibiotic resistance marker. Hence, initial screening for antibiotic resistance should prove a reliable predictor of integration. However, Southern blotting is usually carried out to confirm integration at the desired site as illegitimate integrations have been known to occur. For example, in one study, Southern analysis revealed that approximately 30% of transformants did not contain the desired mutations (Fenno *et al.*, 1993).

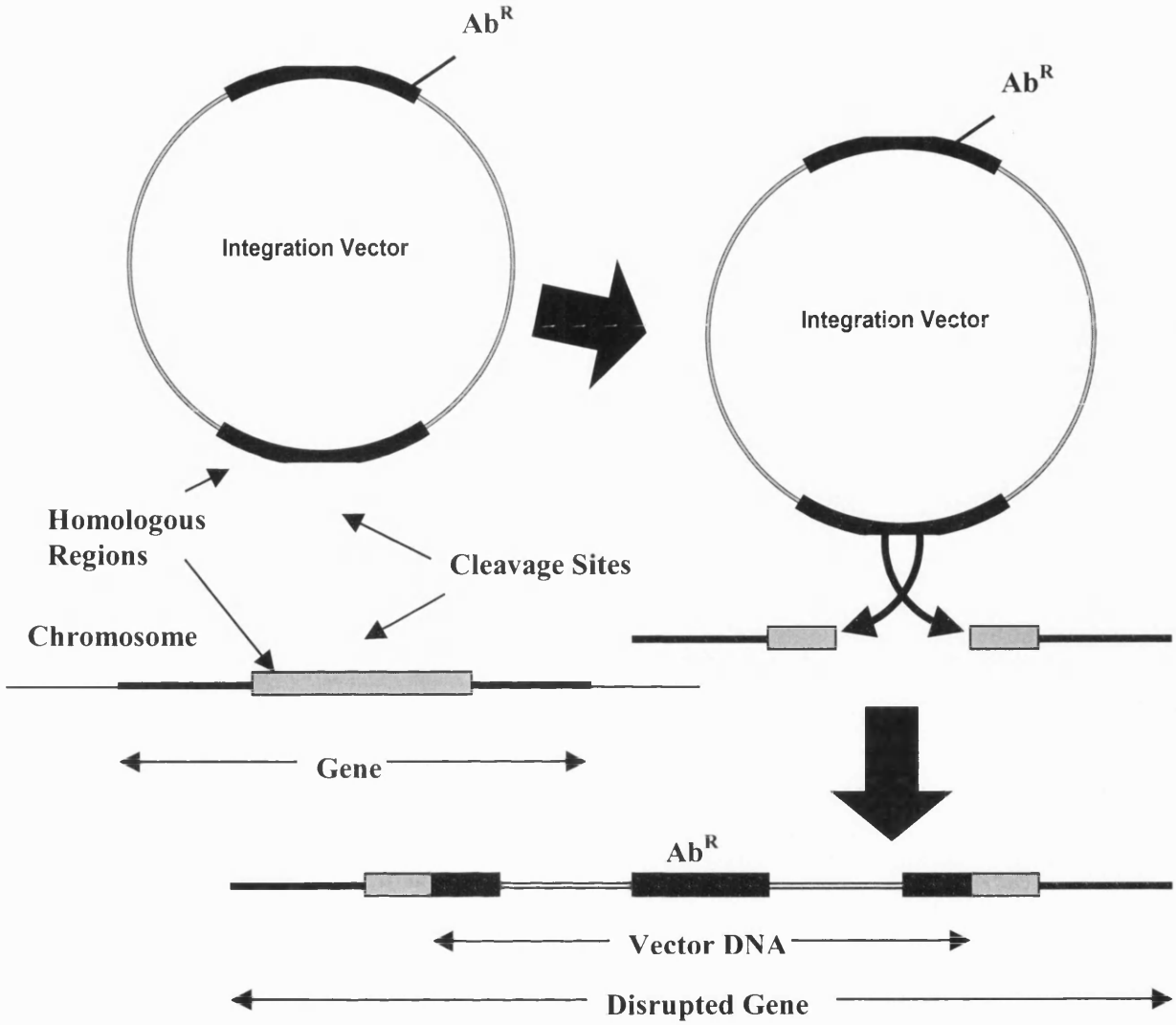


Fig. 3.1A. Campbell-like insertion. A region of the gene of interest (the "target site") is cloned into the integration vector. Single crossover recombination occurs between target sequence on the integration vector and the homologous region on the host chromosome, resulting in insertion duplication of the target site and disruption of the gene through incorporation of the suicide plasmid. This phenomenon can be manipulated to facilitate the inactivation of specific genes. Ab^R is an antibiotic resistance marker.

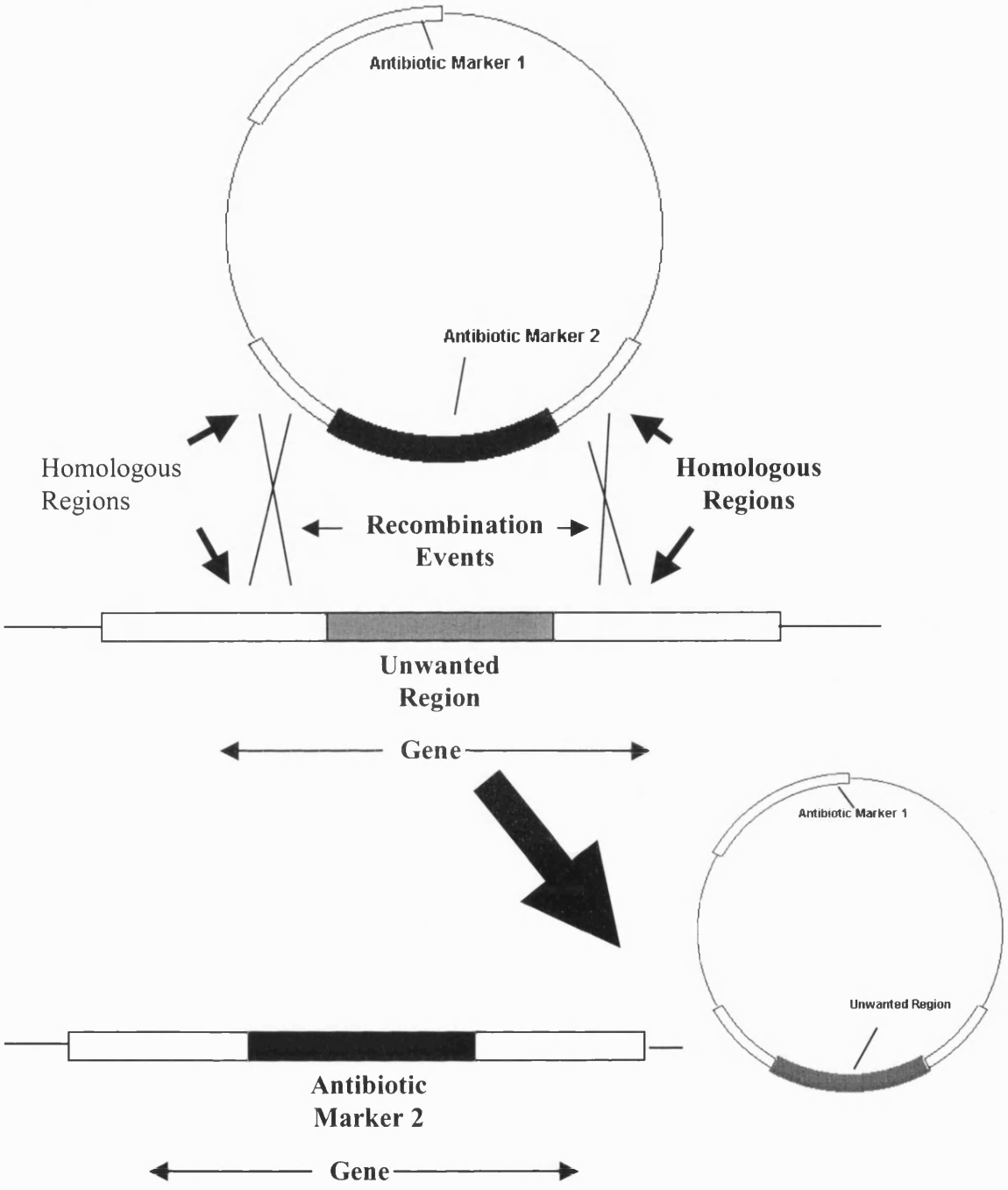


Fig. 3.1B. Schematic of gene inactivation through allelic replacement. A region homologous to that flanking the gene of interest is cloned into the vector. An antibiotic marker is cloned into the target sequence, replacing a portion of the gene of interest. Double crossover recombination occurs between target sequence on either side of the marker and homologous regions on the host chromosome, resulting in exchange of the plasmid-borne marker with an internal fragment of the target gene, thus disrupting the latter. This phenomenon can be manipulated to facilitate the inactivation of specific genes.

Construction of pSF143:*efaA* and pSF143:*efaR*

Bacteria are known to exhibit a species-dependent bias for one particular mode of recombination (Fenno *et al.*, 1993). For example, naturally transformed, double crossover recombination was found to be favoured in *S. pneumoniae* (Pozzi and Guild, 1985) whereas Campbell-like insertion duplication was reported to be preferred in *S. gordonii* Challis DL1 (Fenno *et al.*, 1993). Because a failed attempt had previously been made in our laboratory to inactivate *efaA* by allelic replacement (Flatman, 1999), we speculated that insertion duplication might be more appropriate for *E. faecalis* JH2-2. Indeed, shortly afterwards, insertion duplication mutagenesis was used successfully in *E. faecalis* OG1RF (Singh *et al.*, 1998b).

Another problem with the use of integration plasmid vectors in enterococci is that Gram-positive restriction-modification systems are incompatible with many common *E. coli* plasmids, rendering such plasmids inactive. The suicide vector pSF143 (**Fig. 3.2**) has been used successfully in insertion duplication experiments in streptococci (Tao *et al.*, 1992), which are closely related to the enterococci. Hence, pSF143 was chosen for use in this work. This plasmid has the following characteristics: a) it has an *E. coli* origin of replication, b) it lacks any known streptococcal origin of replication, c) it possesses a streptococcal tetracycline resistance marker (Tc^R) containing an intrinsic promoter element which should facilitate initial screening, d) the Tc^R determinant is also known to work in *E. coli*, and e) it is small at 5.7 kb, which should aid transformation efficiency (Tao *et al.*, 1992).

The length of target sequence required for a successful single cross-over event leading to insertion has been variously quoted as ranging from about 400 or 500 bp to at least 700 or 800 bp (Podbielski *et al.*, 1996). It is widely acknowledged that the efficacy and specificity of cross-over recombination events generally diminishes with decreasing target sequence length provided on the vector. Hence, 503 bp and 743 bp internal fragments of the *efaR* and *efaA* genes (which were about as long as possible for these genes) were amplified by PCR using primer pairs YLL2F/R and YLL8F/R, respectively, from an *E. faecalis* JH2-2 chromosomal DNA template. (These amplicons will henceforth be referred to as "inserts".)

A reservoir of the inserts was created by cloning the gel-purified inserts into the MCS of the plasmid vector pGEM-T, which was then transformed into *E. coli* DH5α by heat shock. Transformants containing recombinant plasmid were identified by blue-white colour selection and the plasmids subjected to restriction digest analysis to confirm insert presence.

During the design of the primers for the inserts, one of each primer pair (YLL2R and YLL4R) had a *Bam*HI restriction site incorporated into its 5' end; the second primer of each pair (YLL2F and YLL4F) had an *Xba*I site added. The restriction sites were chosen because of their presence in the multiple cloning site (MCS) of pSF143. The use of these sites prevented the linearised pSF143 from recircularising without an insert and facilitated the ligation of the inserts into pSF143 in a uniform orientation, resulting in plasmids pSF143:*efaA* and pSF143:*efaR*. Tetracycline-resistant clones were recovered on selective LB agar. Recombinant plasmids were analysed by PCR and restriction digestion, and propagated in *E. coli* DH5α.

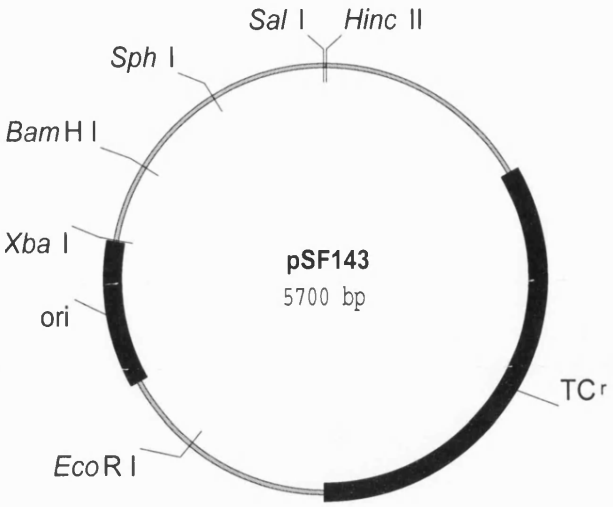


Fig. 3.2. Restriction map of the suicide plasmid pSF143. TC^r is a tetracycline resistance marker; ori is an *E. coli*-derived origins of replication. 743 bp or 503 bp portions of *efaA* and *efaR*, respectively, were ligated into this 5.7 kb plasmid between the *Bam*HI and *Xba*I sites, resulting in plasmids pSF143:*efaA* and pSF143:*efaR*.

Electrotransformation of *E. faecalis* JH2-2

E. faecalis JH2-2 had been reported to transform well with the glycine- and sucrose-using protocol developed by Cruz-Rodz and Gilmore (Cruz-Rodz and Gilmore, 1990), so this protocol was chosen for use. Flatman had previously demonstrated in this laboratory that the plasmid pVA838 could be transformed into *E. faecalis* OG1RF by that protocol (Flatman, 1999). She obtained a transformation frequency of about 1×10^{-6} with *E. faecalis* plasmid preparations to 1×10^{-7} with *E. coli*-derived plasmid. Replication of that work in this study with *E. faecalis* JH2-2 and *E. coli*-derived plasmid yielded similar results (about 1.5×10^{-7} per μg DNA on average), demonstrating the electro-permeability of *E. faecalis* JH2-2. Hence, in this work, electroporation was utilised to facilitate the efforts to inactivate *efaA* and *efaR*. It should be pointed out that the transformation frequencies quoted here are still poor in comparison with those obtained with other bacteria, suggesting that either or both bacterial cell wall and inherent restriction-modification systems are a significant problem.

Initial attempts at electrotransforming *E. faecalis* JH2-2 with pSF143 derivatives were unsuccessful, although the total number of viable cells following electroporation was typically estimated at around 3 x 10^7 cfu/ml. On the basis that tetracycline resistance is mediated by an active efflux pump which is ATP-driven, plates were incubated aerobically rather than anaerobically. This change made no observable impact; controls grew as before, yet no transformants were observed. Up until then, a relatively low concentration of plasmid DNA (approximately 0.1 μg) had been used, as Cruz-Rodz and Gilmore had reported success using as little as 70 ng of DNA (Cruz-Rodz and Gilmore, 1990). Because high DNA concentration (Miller, 1994; Dunny *et al.*, 1991) has been cited by other sources to be an important factor for success (Friesenegger *et al.*, 1991; Miller, 1994), this was strongly suspected to account for the failures. In another paper, Dunny *et al.* observed that 300 ng of DNA was sufficient for transformation (Dunny *et al.*, 1991). However, raising the amount of plasmid DNA to 0.5 μg still yielded no transformants.

The original transformation protocol called for the addition of pre-chilled outgrowth medium following transformation and incubation on ice for 5 minutes prior to incubation at 37°C. In a study performed with *E. coli*, the use of outgrowth medium pre-warmed to 37°C was found to be superior to pre-chilled medium (Antonov *et al.*, 1993). Hence, addition of pre-warmed outgrowth medium was tried instead, and the following incubation on ice step omitted. With these modifications, using 0.5 μg of plasmid DNA and following aerobic incubation, a number of transformants was obtained (transformation efficiency up to 7 x 10^{-7}). Hence, the modified protocol

(i.e. 0.5 μg DNA, pre-warmed outgrowth medium, aerobic incubation of plates) was used in all subsequent electrotransformation attempts.

Verifying Gene Inactivation by pSF143:*efaA /efaR*

As pSF143 lacks an enterococcal origin of replication and hence is theoretically unable to replicate in *E. faecalis* JH2-2, transformants that successfully formed colonies on tetracycline-containing agar plates would be expected to arise from vector-chromosome recombination. Hence, selection for Tc^R should prove a useful initial screen. PCR analysis of total DNA extracts using the primers for *E. faecalis efaR* and *efaA* was attempted in the hope that it would prove a simple and rapid means of screening for insertion duplication mutants. If analysis of the PCR products on an agarose gel yielded one band of approximately 6.5 kb and another of 503 (*efaR*) or 683 bp (*efaA*) band, this could be taken as positive confirmation that insertion duplication had taken place. The drawback of this technique was that if only one band of 503 or 683 bp was seen, no conclusions could be drawn about the success of integration. Such a result could have one of two possible explanations: a) insertion duplication had not taken place, or b) the smaller fragment had been selectively amplified at the expense of the much larger one. Unfortunately, in the event, the results proved inconclusive. Instead, colony blotting with DIG-labelled probe complementary to pSF143, generated by random hexamer labelling, was performed to further screen transformants for more detailed analysis. (**Fig. 3.3**).

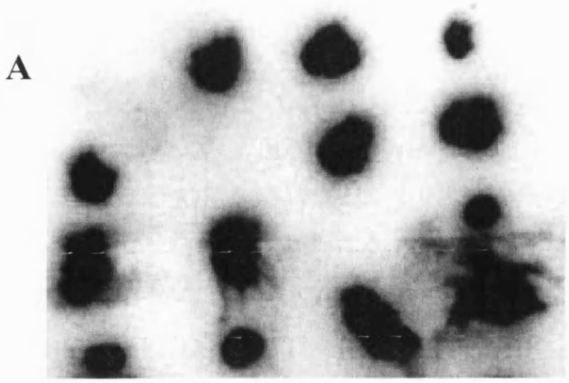


Fig. 3.3. Colony hybridisation analysis with DIG-labelled pSF143 probe (A) was used in the initial screening of potential transformants. The photograph here depicts the analysis of six potential *efaR* mutants. (B) is the key for (A). On this particular membrane, wild-type *E. faecalis* JH2-2 (spots 6 and 11) and a spontaneous *E. faecalis* JH2-2 tetracycline-resistant mutant (spot 1) were used as negative controls. An *E. coli* pSF143:*efaR* colony (spot 16), served as a positive control.

To verify that pSF143 had indeed inserted correctly into the genome, potential *efaR* and *efaA* mutant colonies were tested by Southern analysis. Total cell DNA (approximately 0.5 μg) was extracted and digested with a restriction enzyme which cut once in either plasmid but not within the *efaR* or *efaA* genes. The blots were analysed with DIG-labelled probes complementary to pSF143, *efaA* and *efaR* (**Fig. 3.4**). The results ruled out successful insertion duplication. The pSF143 probe produced additional and unexpected bands, suggesting the possibility of illegitimate integration at sites other than the *efaA* or *efaR* loci. The intensities of some of the bands to which pSF143 hybridised were significantly greater than that of others. In particular, there was a major band slightly smaller than would be expected of linearised insert-containing pSF143.

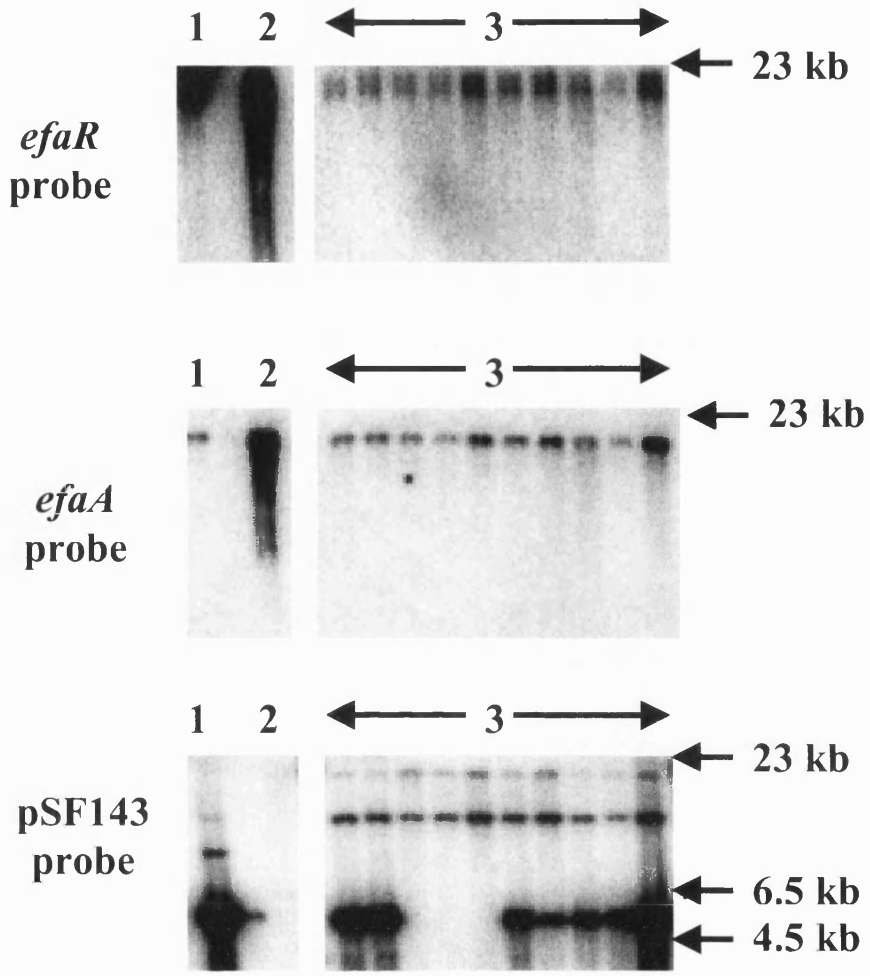


Fig. 3.4. Southern analysis of potential *E. faecalis* JH2-2 pSF143:*efaR*/*efaA* integrants. Neither the *efaR* nor *efaA* probes produced bands expected of successful and accurate integration into the targeted loci. The pSF143 probe produced additional bands of unexpected sizes, with a particularly prominent band around the 6 kb mark, a size corresponding to linear plasmid. Since pSF143 is theoretically unable to replicate in *E. faecalis* other than through integration into the genome, the results suggested illegitimate recombination and multiple plasmid copy integration. Lane 1 contains the genomic DNA of a potential *efaA* mutant, Lane 2 is that of wild-type JH2-2 and Lanes 3 are those of potential *efaR* mutant colonies.

Construction of pKan:*efaA/efaR*

Because of the concerns about the suitability of pSF143's tetracycline resistance marker in *E. faecalis*, and/or possible insertion-duplication at sites other than those intended due to homology between pSF143 and the *E. faecalis* JH2-2 genome, it was decided that insertion duplication attempts utilising pSF143 derivatives should be abandoned in favour of another plasmid with a different antibiotic resistance marker. Because it had been reported as having been used successfully in *E. faecalis* OG1RF (Singh *et al.*, 1998b), the 3 kb plasmid pBluescript SK(-) was chosen (**Fig. 3.5**). For convenience, pBluescript SK(-) will henceforth be referred to as pSK. The small size of the plasmid should aid transformation efficiency, and its possession of an *E. coli* origin of replication afforded perpetuation in *E. coli*. Because of concerns regarding the introduction of genes encoding β-lactamase production into enterococci, a kanamycin resistance marker was obtained from pFW13 and cloned into pSK to replace the existing β-lactamase marker, which was inactivated by truncation.

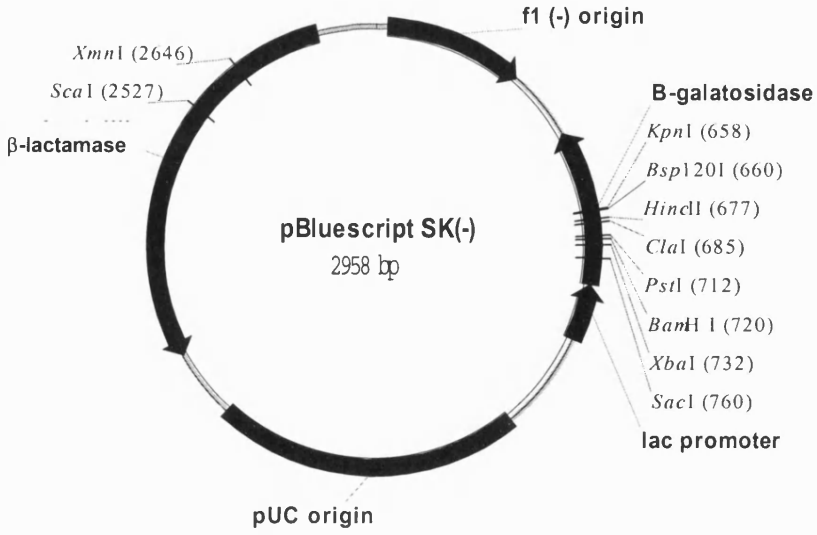


Fig. 3.5. Circular map of the plasmid pBluescript SK(-).

The plasmids pGEM:*efaA* and pGEM:*efaR* were subjected to double digestion with *Bam*HI and *Xba*I or *Pst*I and *Bsp*120I, respectively, to excise their *efaA* and *efaR* inserts. These gel-purified inserts were cloned into similarly-digested pSK vector, generating plasmids pSK:*efaA* and pSK:*efaR*. *E. coli* DH5α colonies potentially containing recombinant plasmids were detected by blue-white colour selection and the plasmids subjected to analysis by restriction digest and PCR to verify the presence of insert. Sequencing was subsequently undertaken using T3 and T7 primers to verify that the insert DNA sequences remained unaltered.

The plasmid pFW13 contains the *aacA* kanamycin resistance determinant. A 1.8 kb *Pst*I/*Xmn*I fragment of this plasmid containing *aacA* and its intrinsic promoter and transcriptional termination elements was excised. The fragment was ligated into a 2.6 kb pSK:*efaA* fragment which had been linearised by restriction with *Sca*I and *Pst*I to create the 4.4 kb pKan:*efaA* (**Fig. 3.6A**). Another 1.8 kb kanamycin resistance cassette, cleaved from pFW13 with *Bsp*120I and *Xmn*I, was ligated into a 2.4 kb fragment of pSK:*efaR* which had been treated with *Bsp*120I and *Sca*I, generating pKan:*efaR* (4.2 kb, **Fig. 3.6B**). In both instances, the restriction of the pSK derivatives resulted in the deliberate inactivation of their β-lactamase markers by significant truncation of those genes. Kanamycin-resistant clones were recovered on selective LB agar and the recovered plasmids subjected to restriction analysis.

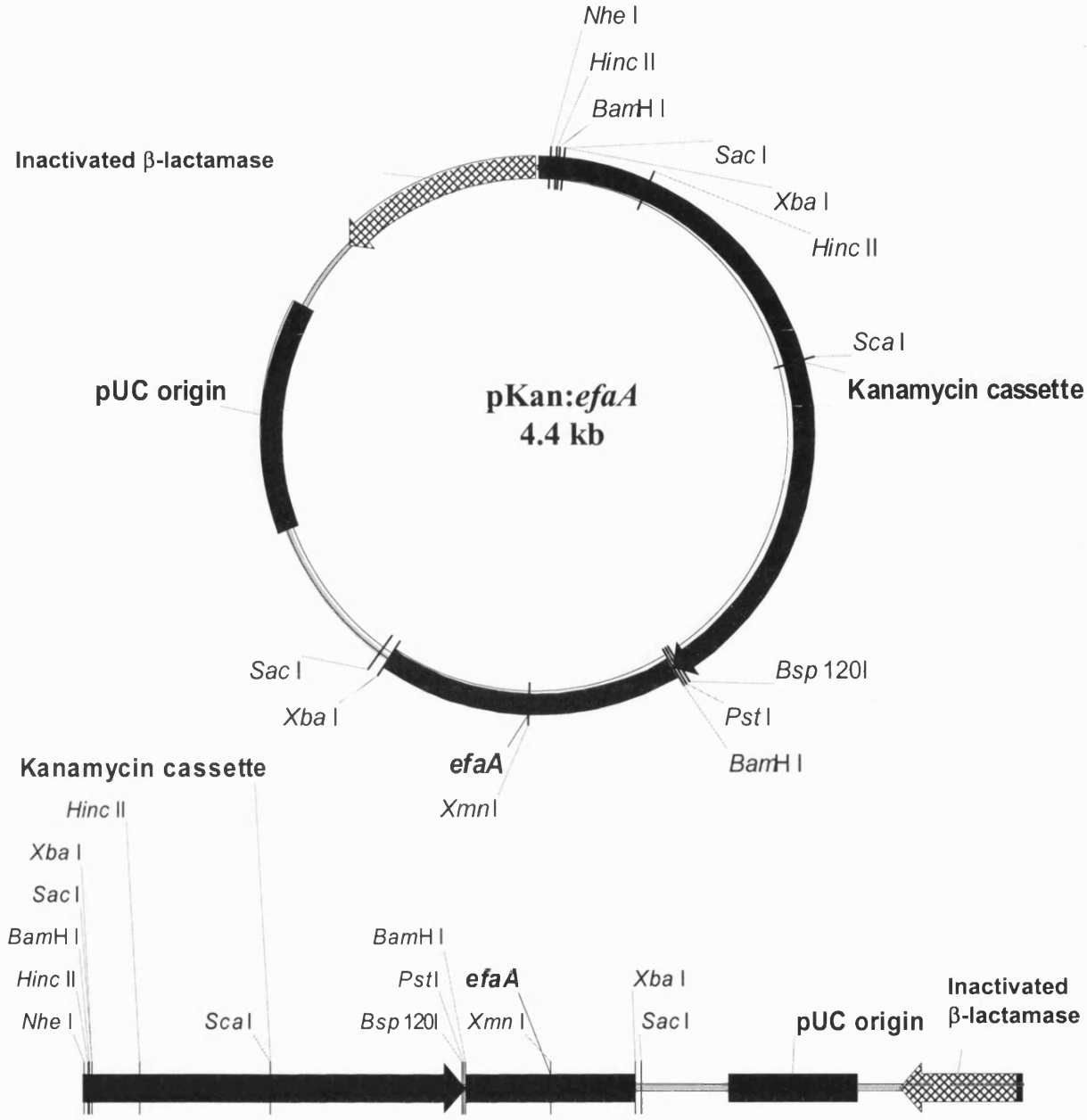


Fig. 3.6A. Circular and linear maps of the pSK-derived integrative plasmid pKan:*efaR*. A 0.75 kb internal fragment of *efaA* was cloned into pSK at the *Bam*HI/*Xba*I site. The 1.8 kb pFW13-derived kanamycin resistance marker was ligated into the plasmid following digestion of the plasmid with *Pst*I and *Sca*I, which also resulted in truncation and deactivation (confirmed experimentally) of the existing β-lactamase marker.

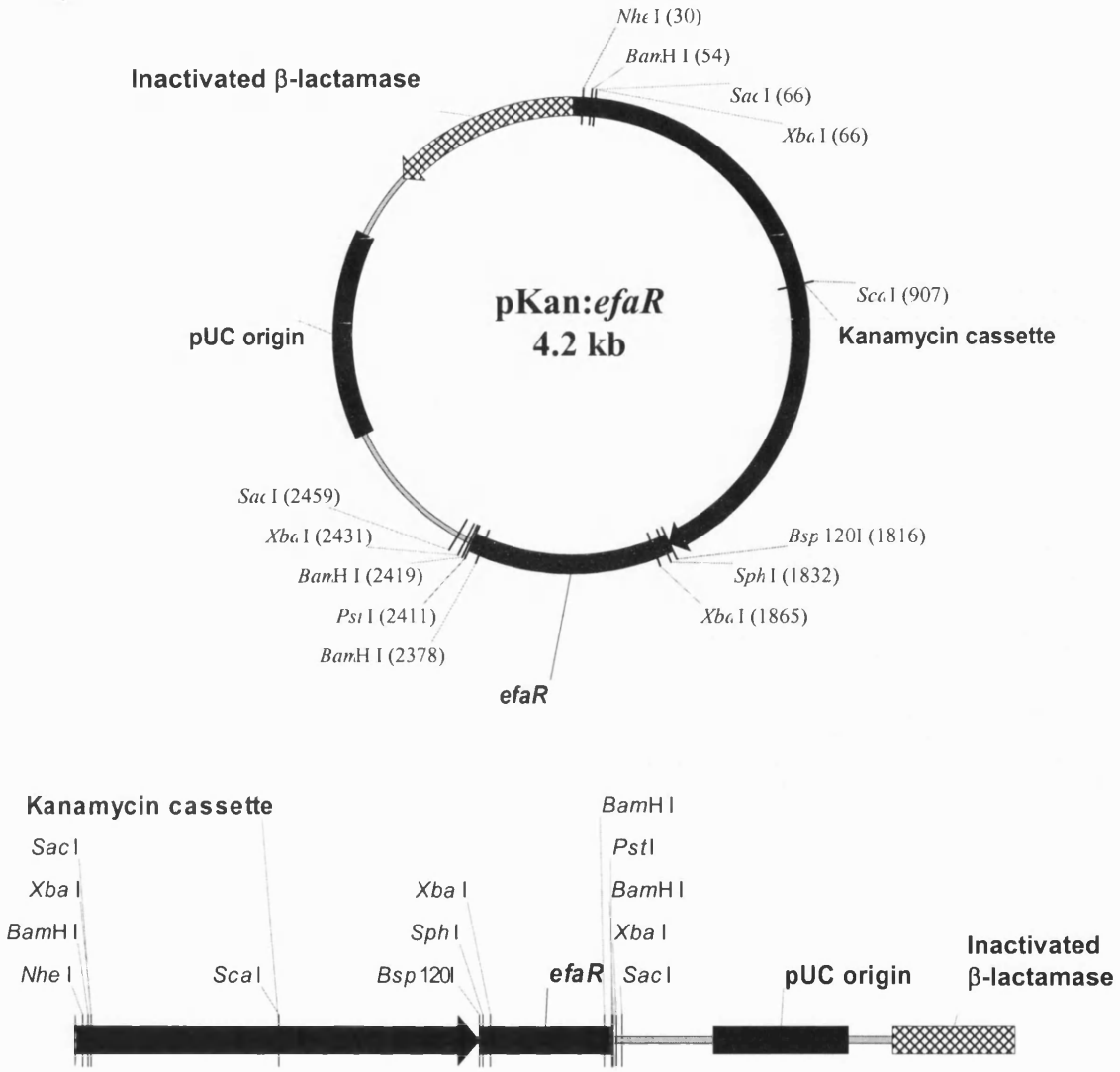


Fig. 3.6B. Circular and linear maps of the pSK-derived integrative plasmid pKan:*efaR*. A 0.5 kb internal fragment of *efaR* was cloned into pSK at the *Pst*I/*Bsp*120I site. The 1.8 kb pFW13-derived kanamycin resistance marker was ligated into the plasmid following digestion of the plasmid with *Bsp*120I and *Sca*I, which also resulted in truncation and deactivation (confirmed experimentally) of the existing β-lactamase marker.

Verifying Gene Inactivation by pKan:*efaA/efaR*

The plasmids pKan:*efaA* and pKan:*efaR* were transformed into *E. faecalis* JH2-2 as previously described for pSF143:*efaA* and pSF143:*efaR*. The colonies recovered were tentatively designated *E. faecalis* JH2-2 pKan:*efaA* and pKan:*efaR*, respectively. Total DNA extracts from kanamycin-resistant transformants recovered on selective LB agar were analysed by Southern blot analysis performed as described in **Methods**. Essentially, the blots were analysed by incubation with DIG-labelled probes generated by PCR for *aacA*, *efaR* or *efaA* as appropriate. Visualisation of bound probe was achieved using anti-digoxigenin alkaline phosphate conjugate with the chemiluminescent substrate CSDP.

Analysis of most *E. faecalis* JH2-2 pKan:*efaR* colonies strongly suggested that insertion duplication had not occurred at the *efaR* locus, i.e. the targeted gene had not been disrupted (**Fig. 3.7**). The *efaR* probe produced two bands of approximately 4.2 kb and 1.5 kb. The intensity of the larger band was considerably greater than that of the smaller, and the size of the larger band corresponded to that of linearised plasmid, whereas that of the smaller band corresponded to that expected of a fragment containing an intact *efaR* gene. The *aacA* probe hybridised to a single band corresponding to the larger band that co-migrates with linearised plasmid. Analysis of an *E. faecalis* JH2-2 pKan:*efaA* colony revealed a similar result.

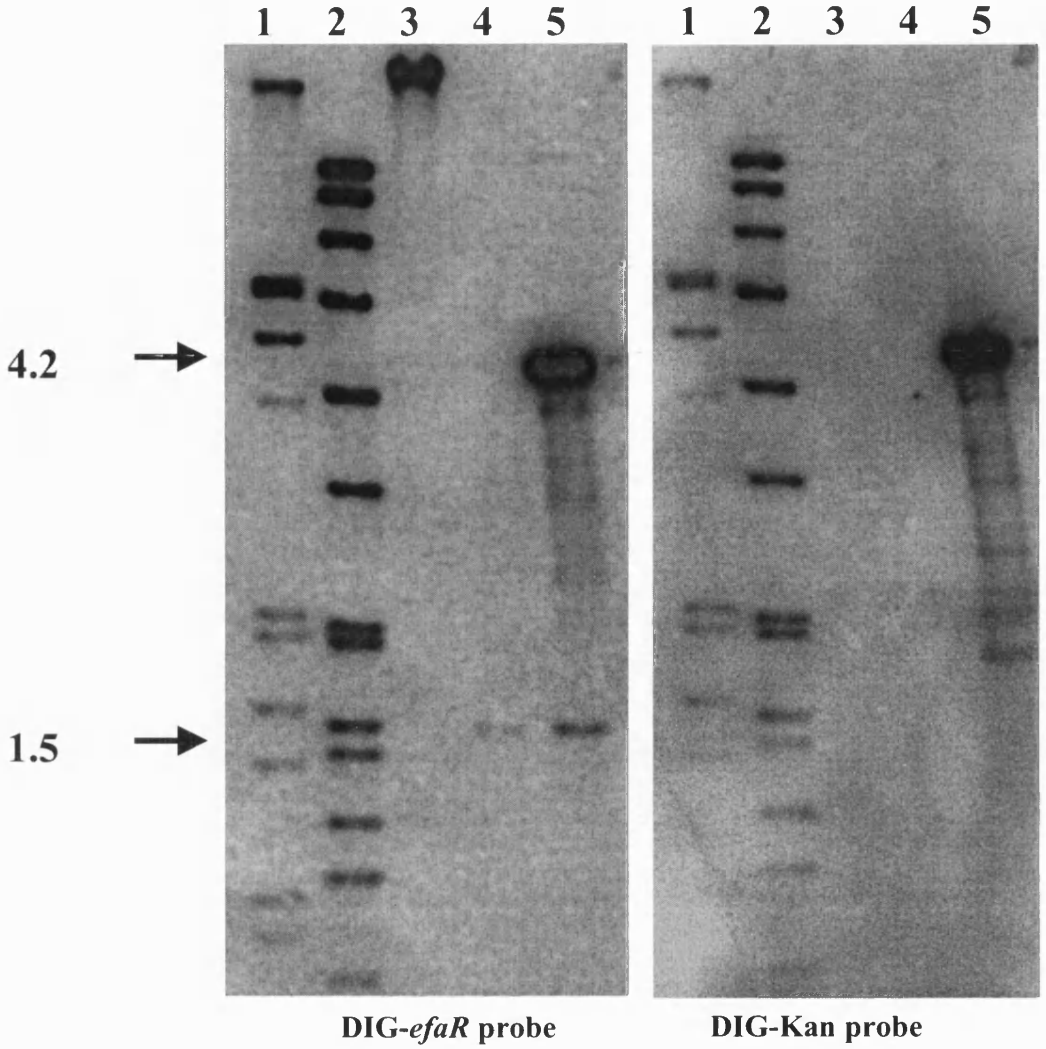


Fig. 3.7. Southern blot analysis of total DNA from wild-type *E. faecalis* JH2-2 and a potential pKan:*efaR* integrant. Lanes 1 and 2 contain molecular weight markers. Lane 3 contains undigested wild-type DNA, whereas Lanes 4 and 5 contain *Nhe*I/*Nru*I-restricted wild-type and transformant DNA fragments, respectively. Following electrophoresis, the DNA was transferred to a positively-charged nylon membrane. The membrane was analysed with DIG-labelled *efaR* probe (on left), then stripped and reprobed with a DIG-labelled probe homologous to an internal region of the kanamycin cassette (on right).

In one particular *E. faecalis* JH2-2 pKan:*efaR* colony, however, Southern analysis unequivocally revealed an illegitimate integration of the plasmid into a part of the genome other than at the *efaR* locus (**Fig. 3.8**). The *efaR* probe revealed three bands, the smallest of which corresponded to that expected of a fragment containing an

intact *efaR* gene. Unlike previous results, the bands were of similar intensity, ruling out multicopy integration in this case. The *aacA* probe hybridised only to one of the two other bands bound by the *efaR* probe, implying that some portion of the *efaR* insert was responsible for integration in this case, albeit not into the intended locus. The *aacA* probe hybridised to DNA derived from this colony but not to DNA derived from wild-type *E. faecalis* JH2-2, clearly demonstrating its foreign origin. This integration event was not a stable one. After three successive passages on non-selective agar, no colonies could be recovered on selective agar. Southern analysis revealed a loss of the kanamycin resistance cassette.

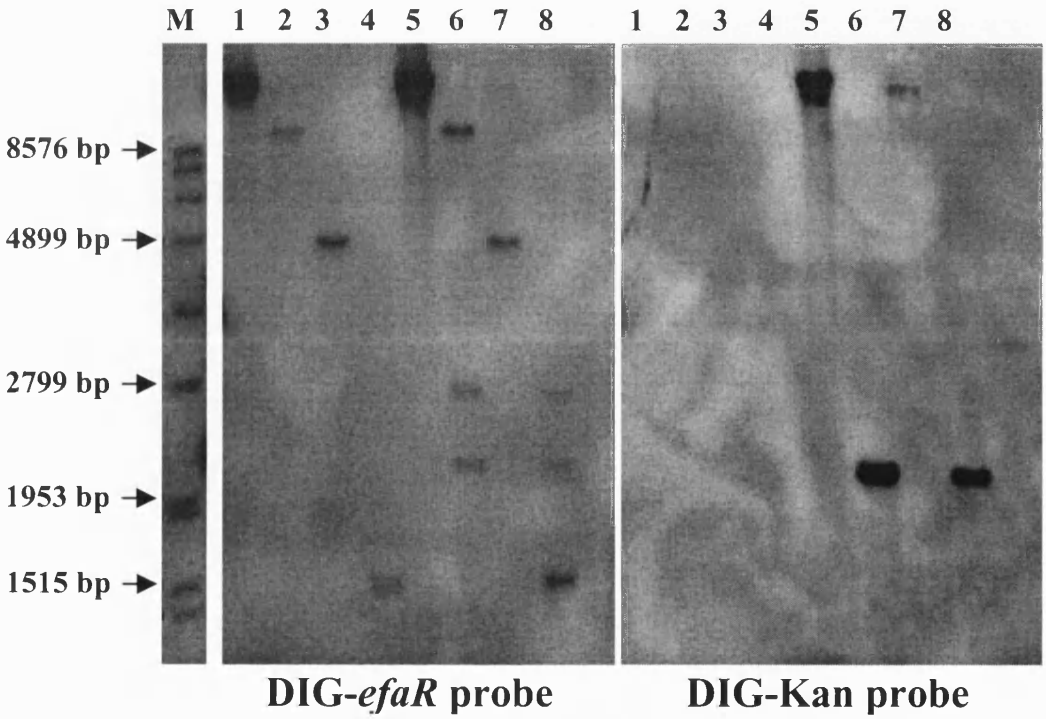


Fig. 3.8. Southern blot analysis of total DNA from wild-type *E. faecalis* JH2-2 and a pKan:*efaR* integrant. Lane M contains a molecular weight marker, with sizes of selected marker bands displayed on the left. Lanes 1 to 4 contain DNA from wild-type *E. faecalis* JH2-2, whereas Lanes 5 to 8 contain DNA from a pKan:*efaR* integrant. The DNA is lanes 1 and 5 were uncut. DNA was restricted with *Nhe*I in lanes 2 and 6, *Nru*I in lanes 3 and 7, and *Nhe*I/*Nru*I-double restricted in lanes 4 and 8. The DNA was transferred to a positively-charged nylon membrane following electrophoresis, and probed with DIG-labelled *efaR* probe (on left). The membrane was then stripped and reprobed with a DIG-labelled probe homologous to an internal region of the kanamycin cassette (on right).

Discussion

Investigation of the traits imparted or controlled by the various genes within the enterococcal genome is essential to the study of the aetiology of enterococcal infection. The inactivation of specific genes can be a powerful tool in facilitating such work. Unfortunately, the use of homologous recombination systems for targeted gene disruption has not been extensively studied in *E. faecalis* thus far, with the majority of *E. faecalis* mutants having been generated by random mutagenesis using transposable genetic elements. Here, because of a previous attempt at targeted mutagenesis by allelic replacement had failed, we have attempted to disrupt the *E. faecalis* JH2-2 *efaA* and *efaR* genes using integrative vectors.

In order to be able to perform insertion duplication mutagenesis in *E. faecalis*, it was necessary first to be able to get the vector into the cell. It is known that as many as forty layers of peptidoglycan can be found in the typical Gram-positive cell wall, and that the thickness of the Gram-positive cell wall renders it less vulnerable to physical stress. As with other Gram-positive cell walls, the enterococcal cell wall is no less significant a physical barrier to entry of large molecules such as DNA into the cell. Studies of enterococcal genes have in the past proven difficult to undertake because of the resistance of the enterococci to traditional methods of introducing modified DNA. With the development of electroporation protocols, and subsequently of methods to enhance further the temporary penetration of the cell wall barrier during electroporation, it became possible to transform many previously "non-transformable" enterococci. However, some *E. faecalis* strains are acknowledged to be untransformable by electroporation even when they are

naturally transformable (Teng *et al.*, 1998). *E. faecalis* JH2-2 proved to be transformable, albeit not very efficiently. In particular, problems were initially encountered trying to recover colonies transformed with pSF143- or pSK-derived suicide vectors. A review of electroporation protocols was conducted and various modifications tried, eventually resulting in a modified protocol (i.e. 0.5 μg DNA, pre-warmed outgrowth medium, aerobic incubation of plates) capable of yielding a small number of transformants. A possible alternative to electroporation, one that could potentially avoid the problems attendant to electroporation of *E. faecalis* and has shown some promise, is conjugation. Shuttle vectors containing an origin of conjugal transfer (*ori*T) can be used to transfer DNA from *E. coli* to Gram-positive species. This method of transferring plasmids was used to inactivate the *E. faecalis* autolysin gene (Teng *et al.*, 1998). Hence, this method of introducing vector DNA into *E. faecalis* JH2-2 should be considered for future attempts at insertion duplication or allelic replacement.

Southern blot and PCR analyses indicated that the insertion-duplication mutagenesis attempts using pSF143- and pSK(-)-derived vectors had failed, as all potential integrants tested retained their native genotype. Southern blot analysis of potential pSF143-derived integrants with DIG-labelled probes complementary to pSF143 suggested the possibility of illegitimate integration at sites other than the *efaA* or *efaR* loci, explaining the unexpected band sizes observed, which could be rationalised as being due to the nucleotide sequence of the flanking genomic DNA. A possible explanation for the greater intensities of the bands corresponding to linearised native pSF143 seen with the pSF143 probe is that following illegitimate integration, further recombinations occurred due to structural instability of the

integrated plasmid, resulting in loss of the inserts and liberation of free plasmid, followed by plasmid replication. This would account for the bands being slightly smaller than expected of linearised plasmid and the lack of corresponding bands with *efaR* or *efaA* probes.

However, in view of the lack of an enterococcal origin of replication in pSF143, that explanation was considered unlikely. The possibility that an *E. coli* origin of replication might enable plasmid replication in *E. faecalis* was rejected as doubtful. A more plausible partial explanation is a phenomenon that has been observed in lactobacilli. Multiple-copy integration of plasmid into the chromosome has been known to occur in *Bacillus anthracis* and lactobacilli when an antibiotic resistance marker is chosen that is not well-expressed in the recipient species (Jenkinson and Jakubovics, personal communication; Sirard *et al.*, 1995). When a single copy of a vector containing a single copy of an antibiotic resistance marker is insufficient to yield resistant clones, integration of additional copies of the suicide plasmid can increase the expression of the resistance marker sufficiently to enable the bacterium to become resistant. High antibiotic concentrations have been deliberately utilised to encourage multiple copy amplification in *B. subtilis* (Meima *et al.*, 1996). The fact that the Tc^R gene was used successfully in streptococci was, unfortunately, not a guarantee that it would work equally well in *E. faecalis*, despite their being closely related. In conjunction with evidence from bacilli that illegitimate intra-plasmid recombination can occur leading to sequence deletion (particularly of foreign DNA sequence) (Meima *et al.*, 1998), multiple copy integration of plasmid provides a more orthodox explanation of the results. In other words, insert deletion via illegitimate intra-plasmid recombination prior to multiple plasmid integration into

the host genome could well account for the out-of-proportion band intensities observed with pSF143 probing and the lack of corresponding bands with *efaR* or *efaA* probes.

Analysis of most of the *E. faecalis* JH2-2 pKan:*efaR* and pKan:*efaA* colonies with DIG-labelled probes raised the possibility that the plasmids remained extra-chromosomal and that *E. coli* origins of replication might be functional in *E. faecalis* JH2-2, enabling replication independent of the chromosome, as did analysis of the pSF143 data. As with the pSF143 data, this interpretation of the data was given due consideration and then rejected as a possible explanation. An alternative interpretation of the intense band observed with the *efaR* probe was that multiple-copy integration into other loci had occurred, since the size of the intense band corresponded to the size of the plasmid. This explanation implies that a single copy of the kanamycin cassette was insufficient to yield kanamycin resistant clones, necessitating the integration of more than one copy of the vector. At least one Gram-positive precedent involving multiple-copy integration of a vector encoding kanamycin resistance does exist, in *B. anthracis* (Sirard *et al.*, 1995). Illegitimate integration did indisputably occur in at least one *E. faecalis* JH2-2 pKan:*efaR* colony, with the data suggesting that some portion of the *efaR* insert was responsible for integration, albeit not into the intended locus.

The fact that homologous recombination at the targeted loci had not been observed whereas illegitimate recombination seems to have occurred at other loci in at least some of the transformants suggested that homologous recombination was not favoured under the experimental conditions used. It may have been that the length of

the target sequences cloned into the suicide vectors was insufficient for homologous recombination at the loci under examination, and/or that experimental conditions favoured illegitimate recombination. The term "illegitimate recombination" refers to recombination between DNA sequences having little or no nucleotide sequence homology, and thus is distinct from homologous recombination, which involves much longer regions of homology. It is believed to be responsible for major genome rearrangements, including deletions, duplications and insertions. Illegitimate recombination has been widely reported in bacterial species ranging from *B. subtilis* (Dempsey and Dubnau, 1989), *Mycobacterium* spp. (McFadden, 1996) and *L. lactis* (Biswas *et al.*, 1993) to *E. coli* (Kusano *et al.*, 1997). In *B. subtilis*, it appears that a minimum of approximately 70 bp of homology is required for detectable RecE-dependent recombination (which is the major homology-dependent recombination system in *B. subtilis*) between plasmid and chromosome (Khasanov *et al.*, 1992). Studies in the same organism have also found that Campbell-like plasmid integration into the host genome can occur either in the absence of any nucleotide sequence homology (Bashkirov *et al.*, 1987), or between regions of short nucleotide homology (6 to 14 bp) (Dempsey and Dubnau, 1989; Bashkirov *et al.*, 1987). The mechanisms by which these forms of illegitimate recombination occur are unclear, but interestingly, Dempsey and Dubnau noted that the short-homology-dependent form can occur in the absence of the RecE recombination system (Dempsey and Dubnau, 1989). Ikeda *et al.* had previously demonstrated in *E. coli* that illegitimate recombination involving sequences containing no homology (short-homology-independent illegitimate recombination) could occur between pBR322 and phage λ (Ikeda *et al.*, 1984; Ikeda *et al.*, 1982) in a process thought to be mediated by DNA gyrase or T4 DNA topoisomerases (Ikeda, 1986). Further work led them to propose

that short-homology-independent illegitimate recombination was mediated by subunit exchange between DNA gyrase, and that in contrast, short-homology-dependent recombination was triggered by double-strand breaks and completed by processing, annealing, and ligation of DNA ends (Shimizu *et al.*, 1997).

The anomalous results obtained in this study may have been because one or more of these forms of illegitimate recombination had inadvertently been favoured by unfortunate choice of *E. faecalis* strain, integrative vector or experimental conditions. Analysis of the sequences of the recombinant DNA junctions should reveal whether this was the case. If so, innovations from similar work in *M. tuberculosis* may provide a solution. Illegitimate recombination has been reported to be a severe problem in this species. It was found that homologous recombination could be enhanced and illegitimate recombination suppressed via the use either of ssDNA generated by alkali denaturation of a dsDNA plasmid template or single-stranded phagemid DNA (Hinds *et al.*, 1999).

Finally, the possibility cannot be discounted that the inability to recover desired mutants was in fact because disruptions of the targeted loci would have been lethal in *E. faecalis* JH2-2.

Chapter 4: Metal Cation Regulation in *Enterococcus faecalis*

Mn^{2+} Regulation of an *E. faecalis* Cluster 9 Operon

Divalent transition metal cations are trace elements essential to the growth and survival of bacteria. The availability of transition metal cations such as Fe^{2+}, Mn^{2+} and Zn^{2+} influences many physiological processes as they have many key roles in cell metabolism. However, high intracellular concentrations of these metal cations can and will interfere with the function of essential proteins. Furthermore, some divalent metal cations, for example cadmium, lead and mercury, are purely toxic and have no known biological role. Therefore, the intracellular concentrations of essential trace elements such as iron, manganese, copper and zinc have to be carefully regulated to sustain a certain supply but nevertheless without poisoning the cell while cations like cadmium and lead have to be kept as low as possible.

The homeostatic maintenance of an optimal intracellular metal cation concentration is achieved via regulation of expression of uptake, storage and excretion mechanisms. Previous workers in this laboratory had succeeded in identifying and cloning from *E. faecalis* and *E. faecium* ABC transporters homologous to members of the cluster 9 family. These have been named EfaCBA and EfmCBA, respectively (Lowe *et al.*, 1995; Flatman, 1999). For convenience, these ABC transporter complexes will henceforth be referred to as Efa and Efm, respectively.

The regulation of expression of divalent transition metal cation uptake and excretion mechanisms is generally achieved at the level of transcription. Metalloregulatory proteins such as DtxR and Fur "sense" the intracellular levels of their cognate ions and modulate transcription of their respective regulons accordingly. As described in the **Introduction**, two main families of metalloregulators responsive to the divalent transition metal cations Fe^{2+}, Mn^{2+} and/or Zn^{2+} have been recognised in bacteria, the classic representatives of which are *E. coli* Fur (ferric uptake repressor) and *C. diphtheriae* DtxR (diphtheria toxin repressor). In addition, in Gram-positive bacteria, there are also the heavy-metal-sensing MerR (mercury resistance repressor) and ArsR (Arsenic resistance repressor) families (Que and Helmann, 2000).

Cluster 9 ABC transporters in *S. epidermidis* and *S. gordonii* have been shown to be regulated by the transcriptional repressors of the DtxR family, SirR (Hill *et al.*, 1998) and ScaR (Jakubovics *et al.*, 2000), respectively . Here, evidence is presented that in conjunction with the divalent transition metal cation Mn^{2+}, another member of the DtxR family, which we have termed EfaR, is responsible for transcriptional regulation of *efaCBA* in *E. faecalis*.

Growth of *E. faecalis* in Manganese-Depleted Medium

In order to study the effects of divalent transition metal cations on the transcription and expression of Efa, it was necessary first to produce a medium depleted of such metal cations. This was achieved by treating BHI or TYHG broth with the styrene

divinylbenzene copolymer, Chelex 100 (Bio-Rad) in accordance with the manufacturer's instructions.

The growth yield of overnight *E. faecalis* JH2-2 cultures grown in Chelex-treated broth supplemented with Mn^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} were compared by measurements of optical density at 600 nm (OD_{600}). Addition of Mn^{2+} increased the yield by nearly 50% ($OD_{600} \approx 0.94 \pm 0.02$) compared with unsupplemented medium ($OD_{600} \approx 0.637 \pm 0.03$). No such effect on growth was seen with any of the other metals tested ($OD_{600} \approx 0.615 \pm 0.01$). These results strongly suggest that Mn^{2+} was the most seriously limiting metal for the growth of *E. faecalis*.

Western Blot Analysis of EfaA Expression

Expression of *efaA* was previously shown to be elevated by growth of *E. faecalis* in medium containing serum (Lowe *et al.*, 1995). Evidence demonstrates that some *efa* homologues, e.g. *sit* (from *S. epidermidis*) (Hill *et al.*, 1998) and *sca* (from *S. gordonii*) (Jakubovics *et al.*, 2000), are manganese-responsive. Unlike *sca*, *sit* expression appears to be responsive to Fe^{2+} as well. To test the hypothesis that Efa expression is manganese-responsive, Western blot analysis was carried out on cell surface protein extracts from *E. faecalis* JH2-2 grown in Chelex-treated BHI supplemented with 10 μM Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} or Zn^{2+}.

The addition of 10 μM Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} or Zn^{2+} to the culture medium had no apparent effect on EfaA production, which remained derepressed. In contrast, when

the medium was supplemented with 10 μM Mn^{2+}, EfaA expression was fully repressed (**Fig. 4.1**). Thus, Efa expression in relation to Mn^{2+} was more like that of *S. gordonii* Sca than *S. epidermidis* Sit.

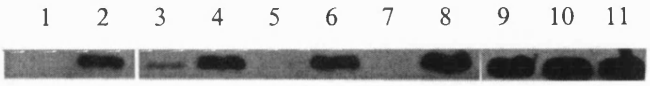


Fig. 4.1. Western blot analysis of EfaA expression. Lanes 1 and 2 contain uninduced and induced *E. coli* XL1-Blue pSK+:GP19, respectively. Induction resulted in expression of EfaA. The other lanes contain *E. faecalis* JH2-2 grown in untreated BHI with untreated glassware (lane 3), in Chelex-treated BHI and untreated glassware (lane 8), and in Chelex-treated BHI and EDTA-treated glassware (lane 4) supplemented with 10 μM Mn^{2+} (lane 5), Fe^{2+} (lane 6), both Mn^{2+} and Fe^{2+} (lane 7), Cu^{2+} (lane 9), Ni^{2+} (lane 10) or Zn^{2+} (lane 11).

Northern Blot Analysis of *efaCBA* Transcription

Having demonstrated that EfaA expression was manganese-responsive, the next step was to determine if manganese-responsive regulation of expression of EfaA was achieved at the level of transcription, as hypothesised. To this end, *E. faecalis* JH2-2 total RNA extracted from bacterial cells grown in Chelex-treated TYHG broth supplemented with 10 μM Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} or Zn^{2} was analysed by Northern blotting.

Hybridisation with ^{32}P-labelled *efaC* probe revealed *efaCBA* to be transcribed as a single polycistronic transcript approximately 2.5 kb in length, as predicted. *S. parasanguis* (Fenno *et al.*, 1995), *S. pneumoniae* (Novak *et al.*, 1998), *S. gordonii* (Kolenbrander *et al.*, 1994; Jakubovics *et al.*, 2000) and some other microorganisms

possess an open reading frame immediately downstream of their *efaCBA* homologues, readthrough of which results in transcripts of approximately 3.2 kb. This fourth gene is absent in *E. faecalis*, in which there is instead a gene (*yfmM*) encoded on the opposite strand. Hence, a 3.2 kb transcript was not detected.

The *efaCBA* transcript was strongly expressed when *E. faecalis* JH2-2 was grown in unsupplemented Chelex-treated broth. Supplementation of the medium with Fe^{3+}, Cu^{2+}, Ni^{2+} and Zn^{2+} had no apparent effect on the level of transcript production, which remained strong. In contrast, on supplementation with Mn^{2+}, *efaCBA* transcript production was either abolished or repressed to below detectable levels (**Fig. 4.2**). The results support the Western blot data and confirm that Efa expression is regulated at the level of transcription in a manganese-responsive manner.

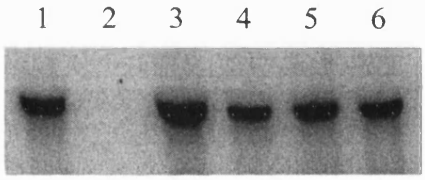


Fig. 4.2. Northern Blot analysis of *efaCBA* transcription. Cells were cultured in chelex-treated BHI. RNA extracts were probed with an internal fragment of *efaC*. Mn^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, or Zn^{2+} were added to 10 μM (lanes 2-6). Lane 1 contained no added metals.

Cloning EfaR, Regulator of Efa Expression

In order to characterise the metal-responsive regulator of Efa expression, this regulatory protein had first to be identified, cloned and over-expressed in order that sufficient quantities be available for experimentation.

Clues as to the identity of the Efa metalloregulator came from *S. gordonii* and *S. epidermidis*. In those bacteria, transcription of the *efaCBA* homologues *scaCBA* (*S. gordonii*) and *sitCBA* (*S. epidermidis*) had been found to be regulated by the DtxR-like repressors ScaR and SirR in a metal-responsive manner (Hill *et al.*, 1998; Jakubovics *et al.*, 2000). In the presence of their cognate metal cations, these metalloregulatory proteins bind to the promoters of cluster 9 operons, thereby preventing transcription. A conserved motif within the promoters, an inverted repeat (IR) resembling the DtxR binding consensus sequence (Lee *et al.*, 1997), is the target for these proteins. Analysis of *E. faecalis efaCBA* promoter nucleotide sequence data revealed two similar inverted repeat sequences within the putative promoter region of the *E. faecalis efa* operon both of which closely resemble the 19 bp DtxR binding consensus sequence (Lee *et al.*, 1997), except that the *E. faecalis* palindromes (like that bound by SirR of *S. epidermidis* (Hill *et al.*, 1998)) lack the central five bases of the consensus, as will be further discussed in **Chapter 5**. Hence, we hypothesised that the manganese regulation of *efa* expression was similarly mediated via a DtxR-like transcriptional regulator. The *E. faecalis* motifs both resemble the DtxR binding consensus sequence and the SirR binding sequence of *S. epidermidis* more closely than they do the ScaR binding sequence of *S. gordonii*.

The unfinished and non-annotated sequence of the *E. faecalis* V583 chromosome at TIGR (http://www.tigr.org) was searched for DtxR-like homologues. A homologue was identified, and the sequence information used to design primers with which to clone the *E. faecalis* JH2-2 DtxR-like homologue, which was termed EfaR (Efa Regulator-of-expression). The primers YLL11F and YLL11R incorporated restriction sites for *Bam*HI and *Nhe*I, respectively (**Table 2.3**). The resulting 672 bp amplimer was cloned into the vector pGEM-T (Promega) to produce pGEM:*efaR*, which was transformed into *E. coli* DH5α. Transformants were recovered on selective LB agar and those containing recombinant plasmid were identified by blue-white colour selection. Recombinant plasmids were subjected to restriction digest analysis and sequencing.

The sequence of the cloned locus was distributed to DDBJ/EMBL/GenBank under Accession No. AF409093. EfaR comprises 222 amino acid residues with a predicted molecular mass of 25.5 kDa. The primary sequence of EfaR is 27% identical (45% similarity) to that of *C. diphtheriae* DtxR, 39% identical (56% similarity) to *S. epidermidis* SirR, 40% identical (57% similarity) to *S. pneumoniae* PsaR and 39% identical (56% similarity) to *S. gordonii* ScaR. Further analysis of the EfaR sequence is undertaken in **Chapter 5**.

The pCal-c vector (Stratagene) was chosen for use in the production of recombinant EfaR. This plasmid enables the expression of cloned proteins as fusions with a C-terminal calmodulin-binding peptide (CBP) tag. The CBP tag, a 4 kDa segment of myosin light chain kinase which binds calmodulin with high affinity in the presence

of low concentrations of calcium, facilitates easy and convenient recovery of the chimeric EfaR-CBP product by affinity-purification from cell lysates using calmodulin affinity resin. After washing away impurities, bound protein could be eluted from the resin in the presence of 2 mM EGTA at neutral pH. The CBP tag contains a recognition site for the site-specific protease thrombin, enabling it to be cleaved by the addition of thrombin and removed by incubation with the calmodulin affinity resin. The plasmid was transformed into *E. coli* BL21(DE3)pLysS and transformants selected on LB agar containing 100 mg/L ampicillin and 50 mg/L chloramphenicol. Sequence integrity was confirmed by sequencing.

The pCal-c protocol called for optimisation of a number of parameters to maximise recombinant protein yield. After some experimentation, the following conditions were found to be optimal: cultures were induced at an OD_{600} of 0.6 with 1 mM IPTG and were harvested after a further 3 ½ hours, when maximal expression had been achieved. It was found necessary to increase the NaCl concentration in the buffers used to 0.3 M to prevent co-purification of contaminating proteins. Protein purification was performed as described in **Methods**. Purified EfaR had an apparent molecular mass of 26 kDa on SDS-PAGE (**Fig. 4.3**).

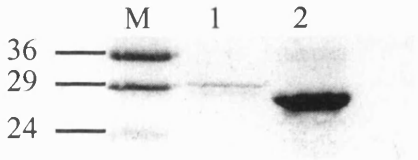


Fig. 4.3. SDS-PAGE of EfaR-CBP fusion protein and EfaR following cleavage of the CBP tag. An EfaR-CBP fusion protein was over-expressed in *E. coli* BL21(DE3)pLysS and purified using calmodulin affinity resin. The CBP tag was cleaved with thrombin and removed by binding it to the affinity resin. EfaR protein was then concentrated using a Vivaspin column as described in **Methods**. The purified proteins were electrophoresed on a denaturing 10% (w/v) polyacrylamide gel and stained with Coomassie Blue. Lanes: M, molecular weight marker (kDa); 1, purified EfaR-CBP; 2, purified and concentrated EfaR following cleavage of CBP.

Electrophoretic Mobility Shift Analysis of EfaR

Previous results had demonstrated that *efaCBA* transcription was metal-responsive, and we speculated that EfaR was the metal-responsive transcriptional repressor responsible for those observations. We further hypothesised that EfaR achieved its repressive effects by binding to the *efaC* promoter. This hypothesis was tested via Electrophoretic Mobility Shift Assay (EMSA). EMSAs were also conducted to determine the cation specificity of EfaR.

DNA fragments in the region of 100-300 bp encompassing the putative promoters of *E. faecalis efaC*, *E. faecalis efaR*, the *E. faecium efaC* homologue *efmC* and the known *S. gordonii scaC* promoter were used as targets in the gel mobility shift assays. Primers YLL14F and YLL14R (**Table 2.3**), both incorporating *Eco*RI restriction sites, were used to amplify 445 bp of *E. faecalis* DNA which was ligated into pGEM-T to produce pGEM:*efaCp*. Cleavage by *Eco*RI and *Hin*cII produced a 131 bp target containing the *efaC* promoter and a 212 bp internal gene fragment for use as a negative control. Primers YLL16F (incorporating a *Hin*cII restriction site)

and YLL16R (*Eco*RI) were used to amplify a 226 bp DNA segment containing the *E. faecium efmC* promoter; the resulting plasmid was designated pGEM:*efmCp*. The *efaR* promoter was amplified using primers YLL15F and YLL15R (*Eco*RI and *Hin*cII, respectively). The 226 bp *scaCp* target DNA has been described by Jakubovics *et al.* (Jakubovics *et al.*, 2000). All sequences were checked by restriction analysis and DNA sequencing. The fragments were excised using *Eco*RI and *Hin*cII and gel extracted, following which the *Eco*RI ends were labelled with [α^{32}P]-dATP using the Klenow fragment of DNA polymerase. Gel mobility shift assays were performed as described in **Methods**.

Most DtxR-homologues, with the notable exception of TroR from *T. pallidum* (Posey *et al.*, 1999), can bind a range of metal cations *in vitro*. Hence, the DNA targets were incubated with varying amounts of EfaR, in the absence of divalent transition metal ions or in the presence of 125 μM Mn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} or Zn^{2+}. EfaR was found to shift *efaCp* in the presence of 125 μM $MnCl_2$ (**Fig. 4.4**). Interestingly, instead of two discrete shift states that had been predicted to occur at intermediate EfaR concentrations, a gradual increase in retardation of DNA migration was observed with increasing EfaR concentration between 0.1 and 0.5 μM. Of the other metals tested, Co^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} also retarded DNA migration (**Fig. 4.5**). Inclusion of EDTA, a divalent metal ion chelator, was found to prevent the Mn^{2+} shift (**Fig. 4.6**). EfaR was also able to shift *efmCp* (**Fig. 4.7**) but did not shift either *scaCp* or *efaRp* (**Fig. 4.8**). The latter suggested that *efaR* is not autoregulated. On the other hand, *S. gordonii* ScaR was able to shift *scaCp*, *efaCp* and *efmCp* in the presence of Mn^{2+} (**Fig. 4.9** and **Fig. 4.10**).

EfaR Protects Two Regions of the *efaC* Promoter from DNase I Digestion

To determine the exact nucleotide sequence bound by EfaR, DNase I footprint analysis was performed on the same *efaCp* and *efmCp* fragments used in the EMSAs. DNase I footprint analysis was performed as described by Jakubovics *et al.* (Jakubovics *et al.*, 2000). Protein-DNA binding was carried out for 15 min at 30°C, following which DNase I (0.1 unit) was added and the incubation continued for a further 1 min. The DNA fragments were separated on a denaturing polyacrylamide gel and analysed by autoradiography. The sizes of fragments were estimated by comparison with a Maxam and Gilbert A+G sequencing ladder (Sambrook *et al.*, 1989).

Binding of EfaR to *efaCp* resulted in the protection of two approximately 21 bp regions of dyad symmetry (**Fig. 4.11**) straddling the -35 and -10 elements (termed boxes I and II; box I is just upstream of the putative -35 element while box II is downstream of box I and just upstream of the putative ribosome binding site). Some protection of the region between the two boxes was also noted.

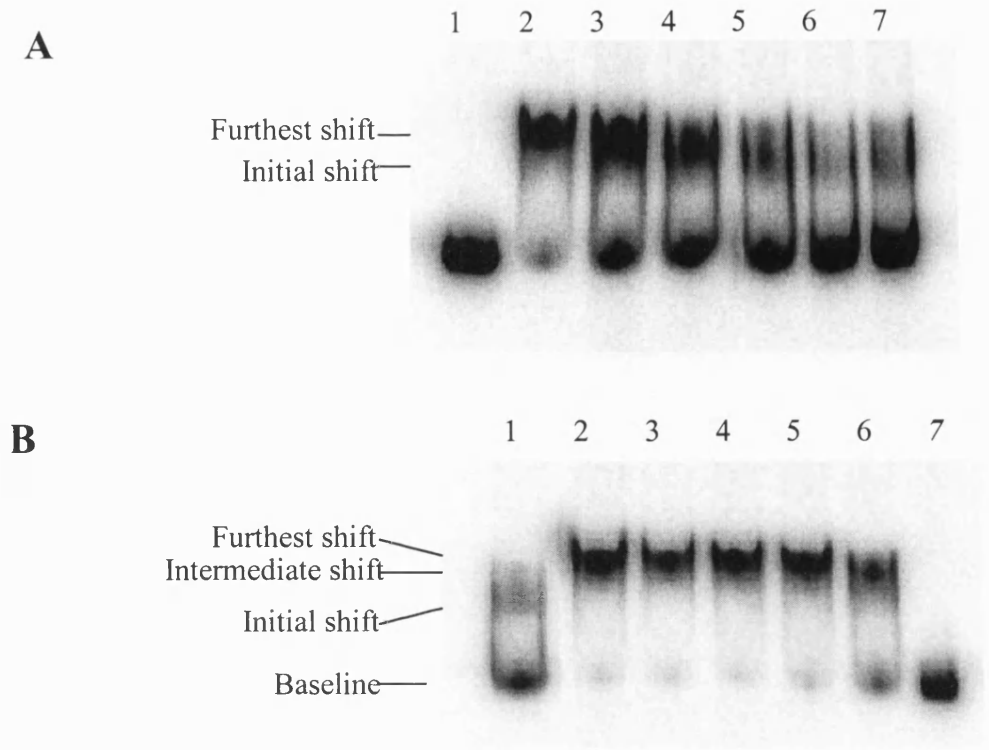


Fig. 4.4. Electrophoretic mobility shift analysis of binding of EfaR to the putative *efaC* promoter (*efaCp*). Purified EfaR was incubated with [α^{32}P]-labelled *efaCp* (lane 1) and 125 μM Mn^{2+}. (A) Lanes 1 to 7 contain EfaR concentrations of 0, 0.5, 0.3, 0.2, 0.1, 0.08, and 0.06 μM, respectively. (B) Lanes 1 to 7 contain EfaR concentrations of 0.1, 1.0, 0.8, 0.6, 0.4, 0.2, and 0 μM, respectively.

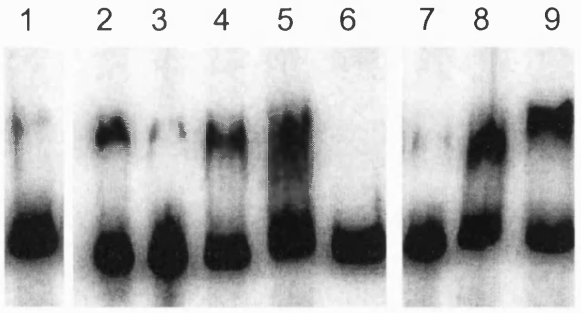


Fig. 4.5. Electrophoretic mobility shift analysis of EfaR binding to *efaCp*. Purified EfaR was incubated with [α^{32}P]-labelled *efaCp* and 125 μM Mn^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} or Cu^{2+} (lanes 2 to 5 and 7 to 9, respectively). Lane 1 contained no added cations while lane 6 contained no EfaR.

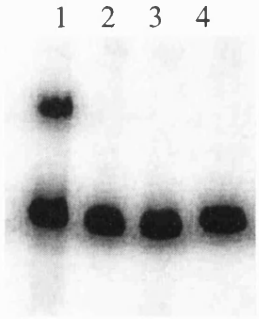


Fig. 4.6. Electrophoretic mobility shift analysis of effect of EDTA on EfaR binding to *efaCp*. [α^{32}P]-labelled *efaCp* was incubated with EfaR (lanes 1, 3 and 4) and Mn^{2+} (lanes 1 to 3). EDTA was added to lane 3. Lane 2 contained no EfaR, whilst lane 4 contained no Mn^{2+}.

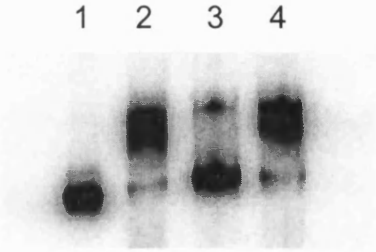


Fig. 4.7. Electrophoretic mobility shift analysis of EfaR binding to *E. faecium efmCp*. Purified EfaR was incubated with [α^{32}P]-labelled *efmCp* and Mn^{2+} (lanes 2 to 4). Additionally, unlabelled *efmCp* and control fragment were included in lanes 3 and 4, respectively. Lane 1 contains no protein.

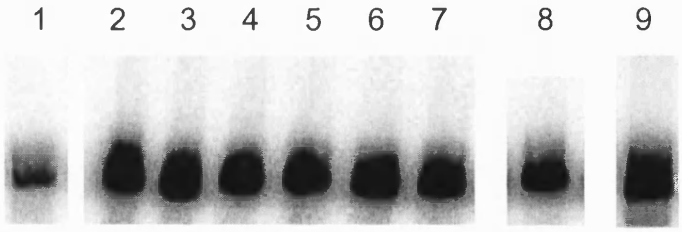


Fig. 4.8. Electrophoretic mobility shift analysis of EfaR binding to *S. gordonii scaCp*, *E. faecalis efaRp* and control fragment. Purified EfaR was incubated with [α^{32}P]-labelled *scaCp* and Mn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} or Zn^{2+} in lanes 1 to 7, respectively. EfaR was also incubated in the presence of Mn^{2+} with *efaRp* or control fragment in lanes 8 and 9, respectively.

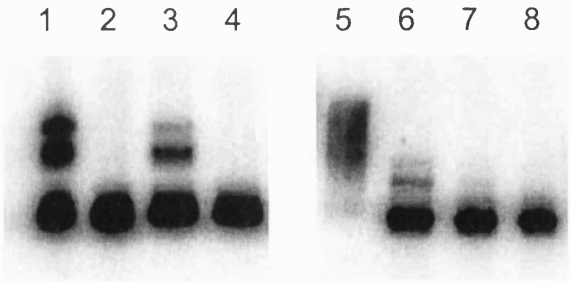


Fig. 4.9. Electrophoretic mobility shift analysis of *S. gordonii* ScaR binding to *S. gordonii scaCp* and *E. faecium efmCp*. Lanes 1 to 4: 0.1 μM ScaR was incubated with [α^{32}P]-labelled *scaCp* in the presence (lanes 1, 3 and 4) or absence (lane 3) of Mn^{2+}. Lane 2 contained no protein. EDTA was added to lane 4. Lanes 5 to 8: [α^{32}P]-labelled *efmCp* was incubated with 1.0, 0.1, 0.01 and 0 μM ScaR, respectively.

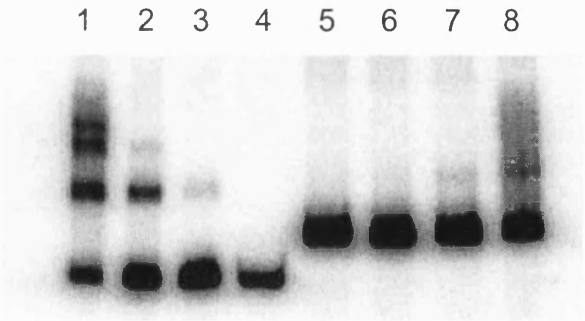
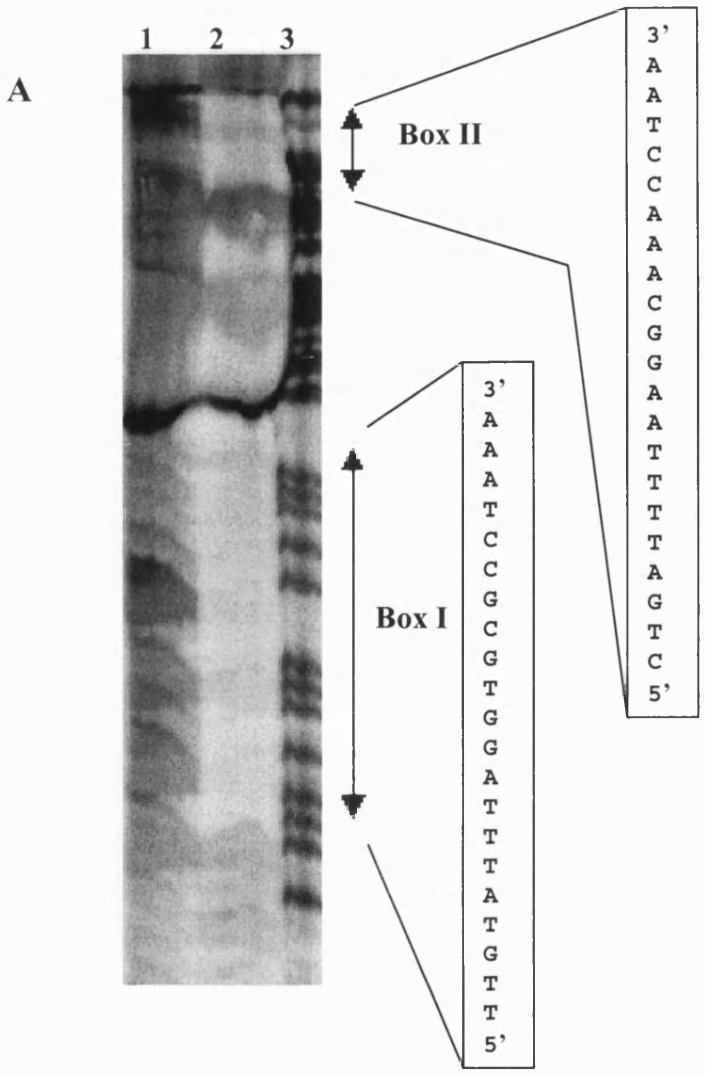


Fig. 4.10. Electrophoretic mobility shift analysis of ScaR binding to *E. faecalis efaCp* and control fragment. Purified EfaR (1, 0.1, 0.01 and 0 μM) was incubated with [α^{32}P]-labelled *efaCp* and Mn^{2+} in lanes 1 to 4, respectively. EfaR (0, 0.01, 0.1 and 1 μM) was incubated with [α^{32}P]-labelled control fragment and Mn^{2+} in lanes 5 to 8, respectively.



B

5'GCTTGTAT**TTAGGTGCGCCTAA**AAATTAT**TTGCAT**TTTCTTAAACTA
Box I -35

TCCCT**TATACT**GATTT**TAAGGCAAACCTAA**AAAA**GGAGG**AATTTC**ATG** 3'
-10 Box II RBS

Fig. 4.11. (A) DNase I footprint of the putative *efaC* promoter region with 0.5 μM EfaR. The DNA was digested with DNase I and fragments were analysed in the absence or presence of EfaR (lanes 1 and 2, respectively) on a denaturing 6% (w/v) polyacrylamide gel, and compared with a Maxam and Gilbert A+G ladder (lane 3). Two regions containing inverted palindromes, termed boxes I and II (both 21 bp, respectively), were protected from digestion by DNase I. Some degree of protection also appeared to be afforded to the region between the two boxes. (B) DNA sequence of putative *efaC* promoter. Nucleotides protected by footprinting are denoted by arrows. Putative EfaR boxes, -35 and –10 elements and ribosome binding site (RBS) are also indicated.

Discussion

Western blot analysis demonstrated that EfaA expression is strongly repressed by Mn^{2+}. Addition of Mn^{2+} to the growth medium abolished EfaA expression. In contrast, the addition of iron, cobalt, nickel or zinc had no apparent effect on EfaA expression. The results suggest that the induction of EfaA and ScaA observed following growth in serum-supplemented medium (Kolenbrander *et al.*, 1998; Lowe *et al.*, 1995) was likely due to sequestration of Mn^{2+} from the growth medium by components present in serum. Northern analysis correlated with the Western data, revealing that *efaCBA* transcription was repressed by Mn^{2+}. Transcription was abolished by supplementation of the growth medium with Mn^{2+} but not with Fe^{2+}, Cu^{2+}, Ni^{2+} or Zn^{2+}. The results confirm that Efa expression is indeed regulated at the level of transcription in a manganese-responsive manner. The relationship between Mn^{2+} availability in the medium and Efa expression is consistent with a role for Mn^{2+} in the regulation of *efa* expression. Although in extensive mutagenesis studies we have not yet been able to isolate an *efaA* mutant, our cumulative results so far, taken together with findings from the Efa homologues Sca (Kolenbrander *et al.*, 1998) and Psa (Dintilhac *et al.*, 1997b), provide evidence that Efa is a manganese permease.

The strong repression exerted by manganese on Efa expression and *efaCBA* transcription was intriguing, considering that its effect on Sca expression was far less complete. For example, transcription of *scaCBA* was reduced by approximately 20-fold when the concentration of Mn^{2+} in the medium was increased from 0.1 to 50 μM Mn^{2+} (Jakubovics *et al.*, 2000). One explanation is the presence of two

binding sites in the *efaCBA* operon compared with one in *scaCBA*. Assuming that binding to either one by EfaR is sufficient to prevent RNA polymerase binding, and that repressor binding is dynamic, the chances of the promoter being occupied by a repressor unit would be higher with two binding sites. A second possibility is that the *efaCBA* transcription start site may lie within the footprint of EfaR whereas that of *scaCBA* is known to lie outside the ScaR footprint (Jakubovics *et al.*, 2000). The former may hinder RNA polymerase access to the promoter more effectively.

Electrophoretic mobility shift assays and DNase I experiments confirmed the hypothesis that the *E. faecalis* DtxR-like protein EfaR binds the inverted repeat (IR) elements of the *efaC* promoter in the presence of Mn^{2+}. These experiments, together with published evidence from experiments with homologues, strongly suggest that the DtxR-like protein is the regulator of expression of *efa*. Hence, we have designated this protein EfaR (Efa Regulator-of-expression), and termed the IR elements EfaR boxes. However, we have not been able to isolate an *efaR* mutant with which to perform comparative experiments.

Interaction between EfaR and the *efaC* promoter DNA was expected to result in two discrete bands (shift states) at intermediate EfaR concentrations, due to the occupation of one of the two boxes on some DNA fragments by the dimeric EfaR holorepressor and occupation of both boxes on others. In practice, only a single band was observed at all concentrations tested. However, it was observed that the retardation of the DNA band increased with increasing EfaR concentration between 100 nM and 500 nM, with the binding sites presumably becoming saturated at the latter concentration. Why a gradual shift should have been observed rather than two

discrete shift states is not clear. A possible explanation is that EfaR bound as separate monomeric units rather than as dimeric holorepressor units, with more monomeric units being recruited as EfaR concentration increased, giving the impression of a more gradual shift. Alternatively, one dimeric holorepressor unit bound initially, with further units than polymerising along the DNA to the other binding site in a manner analogous to that which has been proposed for Fur (Le Cam *et al.*, 1994). A third possibility is that an intermediate double shift state occurred transiently when EfaR concentration was somewhere between 0.1 and 0.2 μM. At 0.2 μM EfaR, both binding sites became saturated, a point labelled "intermediate shift" in panel B of **Fig. 4.4**. As EfaR concentration increased further, more EfaR holorepressor molecules either polymerised to or became trapped between the already-bound repressor complexes, thus further retarding DNA migration and affording some protection from DNase I digestion to the inter-box region, labelled "furthest shift" in panel B of **Fig. 4.4**.

The promoter region of *efaR* contained what appears to be one half of an EfaR box, but the results of EMSA analysis suggests that auto-regulation is unlikely. EfaR was also unable to bind to the streptococcal promoter *scaCp*, but in contrast, the *S. gordonii* DtxR-like protein ScaR bound *efaCp* and *efmCp*, producing the multiple discrete shifts characteristic of its interaction with *scaCp*. In EMSAs involving *S. gordonii* ScaR, two bands were typically observed (or three, with very high protein concentrations) here and in previously-published work (Jakubovics *et al.*, 2000). ScaR produced the same number of shifts with all three promoter fragments.

The discrete EfaR footprints on *efaCp* (approximately 21 bp each) appear to be smaller individually than those reported for ScaR with *scaCp* (approximately 46 bp) by Jakubovics *et al.* (Jakubovics *et al.*, 2000), if the partial protection of the region in between the two EfaR boxes is not considered. The DtxR consensus sequence is considerably better conserved in the promoters of *E. faecalis* than in the streptococci (discussed further in **Chapter 5**). In fact, the binding sequences in *efaCBA* closely resemble those of the *S. epidermidis sit* operon and IRP1 and IRP4 of *C. diphtheriae* in that they all lack the central five bases of the consensus. In contrast, the permease operators of streptococci contain a highly conserved AT-rich palindrome which is poorly conserved in *E. faecalis*, *S. epidermidis* or *C. diphtheriae*. The size of the ScaR footprint size could well be the result of ScaR binding to this palindrome rather than, or in addition to, the DtxR-like binding motif. The fact that EfaR would not bind to the *scaC* promoter implies that only streptococcal DtxR homologues are able to bind to this palindrome. This may account for the differences between ScaR and EfaR-mediated EMSA shifts

Despite the apparent high overall conservation of domain structure and metal ion-binding residues across the DtxR family of proteins, the various DtxR homologues have different metal ion preferences *in vivo*, although most can bind a wide range of metal cations *in vitro*. DtxR, IdeR and SloR (Spatafora *et al.*, 2001) are specific for Fe^{2+}, while SirR responds to Fe^{2+} and Mn^{2+} (Hill *et al.*, 1998). ScaR and TroR respond only to Mn^{2+} (Posey *et al.*, 1999; Jakubovics *et al.*, 2000). EfaA expression and *efaCBA* transcript production were sensitive only to Mn^{2+}, which suggests that EfaR may be Mn^{2+}-specific *in vivo*, although like its homologues, it was able to bind a range of divalent cations *in vitro*. Possible structural reasons behind the apparent

differences in metal cation specificity of these homologues are examined in more detail in **Chapter 5**.

Divalent transition metal ions such as manganese, iron and zinc are essential both for the growth and survival of microorganisms and yet can they also be potentially toxic at high concentrations. Hence, careful regulation of their intracellular concentrations is essential. Aside from a few exceptions like *L. plantarum* (Weinberg, 1997; Archibald, 1983) and *Borrelia burgdorferi* (Posey and Gherardini, 2000), iron is essential to all known microorganisms, and particularly in the pathogenesis of infections (Vasil and Ochsner, 1999; Crosa, 1997). It appears that Mn^{2+} can be utilised by certain streptococci in the absence of Fe (Martin *et al.*, 1984; Niven *et al.*, 1999; Spatafora and Moore, 1998), an ability which would give those microorganisms a selective advantage where iron availability is restricted, e.g. in the human host. Manganese is required by sporulating bacteria during sporulation, in the production of secondary metabolites and various antigens and toxins and as a co-factor for numerous enzymes. Examples include Mn-catalase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Very recently, CpsB, a phosphotyrosine-protein phosphatase belonging to the PHP (polymerase and histidinol phosphatase) family of phosphoesterases which plays a key role in the synthesis of capsular polysaccharide essential for pneumococcal virulence, was shown to be manganese-dependent (Morona *et al.*, 2002). The importance of manganese in bacteria in relation to combating oxidative stress is becoming increasingly recognised (Jakubovics and Jenkinson, 2001). For example, in *S. pneumoniae*, it has very recently been demonstrated that deactivation of the *efaA* homologue *psaA* results in decreased superoxide dismutase activity and hypersensitivity to oxidative stress

(Tseng *et al.*, 2002). The *psaBCA* operon, like *efaCBA*, is thought to encode a manganese transporter, and manganese is essential for the detoxification of reactive oxygen species (ROS) in most bacteria, principally as a cofactor for manganese-cofactored superoxide dismutase (MnSOD). In fact, the activity of MnSOD in *Streptococcus suis* can be reduced simply by manganese-limitation (Niven *et al.*, 1999). Manganese may be more important than iron in the pathogenesis of *T. pallidum* infections as its genome contains few or no ORFs encoding likely Fe-containing proteins. (Posey *et al.*, 1999). Since the addition of manganese, but not iron, increases the growth yield of *E. faecalis*, Mn^{2+} is likely an important micronutrient for this microorganism. We hypothesise that Efa might function as a high-affinity manganese permease in *E. faecalis*, possibly playing a role in the infection of human host tissues, where Mn^{2+} availability can be as low as 20 nM (Krachler *et al.*, 1999).

Mn^{2+}-specific metalloregulation has been documented in a number of bacterial species, e.g. *S. gordonii* (ScaR), *T. pallidum* (TroR) and *B. subtilis* (PerR and MntR). ScaR and TroR appear to function as classical metal-dependent repressors. Both modulate the expression of ABC metal permeases. In contrast, MntR appears to be a bifunctional regulator which represses a low-affinity proton-coupled Mn^{2+} transporter (*mntH*) in high Mn conditions and synergistically activates a Mn-specific ABC transporter system (*mntABCD*) under low Mn conditions. PerR binds peroxide stress genes in the presence of Fe^{2+} or Mn^{2+} (Bsat *et al.*, 1996) and represses the expression of these genes. The accumulation of superoxide radicals causes PerR to dissociate from target operators by mechanisms as yet not understood, resulting in induction of genes encoding products such as catalase, the H_2O_2 stress-response

DNA-binding protein MrgA, alkylhydroperoxide reductase (AhpCF) and haem biosynthesis enzymes (Bsat *et al.*, 1998). Bsat *et al.* proposed that peroxide stress activation of PerR was linked to the bound metal ion with either a change in the oxidative state of the complexed metal or a metal-catalysed oxidation reaction that damages PerR (Bsat *et al.*, 1998). Mechanistically, this would separate PerR from OxyR, which is activated by H_2O_2-catalyzed disulfide bond formation. Freely-ionic Mn^{2+} can also perform an indirect role in the alleviation of oxidative stress by quenching peroxyl radicals to form stable hydroperoxides. Hence, Fe^{2+}- and Mn^{2+}-responsive metalloregulators are often found to be associated with Mn^{2+}-specific transport and oxidative stress.

On the basis of our findings, we propose a model for EfaR metalloregulation of Efa in *E. faecalis* wherein Mn^{2+} acts as a co-repressor. According to this model, when Mn^{2+} is abundant, intracellular Mn^{2+} levels rise resulting in the formation of EfaR-Mn^{2+} complexes which bind to the *efa* promoter, inhibiting transcription and hence reducing Mn^{2+} uptake. When freely-available Mn^{2+} is scarce on the other hand, e.g. in human serum, the apoprotein cannot bind the *efa* promoter, derepressing *efa* expression and hence increasing Efa permease levels, aiding Mn^{2+} scavenging. In addition to regulating metal ion transport, *C. diphtheriae* DtxR is also known to regulate the expression of virulence genes whose products include adhesins and a toxin (Tao *et al.*, 1994). EfaR boxes have also been found in the promoters of other *E. faecalis* genes (**see Chapter 5**), for example Natural Resistance-Associated Macrophage Proteins (NRAMP) homologues, suggesting that EfaR, like DtxR, may have a global regulatory role.

Chapter 5: Computer-based Sequence Analyses

In this chapter, comparison of the amino acid sequence of EfaR with sequence databases is described. Examination of the promoters of various *E. faecalis* genes is performed, to identify possible EfaR recognition sites. The data presented here will provide an explanation of the results described in the previous chapter and provide further insights into the role of EfaR as a metal-responsive global regulator in *E. faecalis*.

EfaR Nucleotide and Protein Sequence Analyses

The nucleotide sequence of the *E. faecalis* JH2-2 *efaR* promoter region and open reading frame was determined by sequencing and the result shown in **Fig. 5.1**. The sequence has been distributed to DDBJ/EMBL/GenBank under Accession No. AF409093.

There is a potential Shine-Dalgarno ribosome binding site (GGATGA) located 7-12 bp upstream of the ATG start codon. Translation of the 666 bp *efaR* open reading frame revealed a 222 amino acid peptide (**Fig. 5.1**) with a predicted molecular mass of 25,485.7 kDa and a theoretical isoelectric point of 4.86. The protein is generally hydrophilic, with a GRAnd AVerage of HydropathicitY (GRAVY) score of -0.421 as calculated by the method of Kyte and Doolittle (Kyte and Doolittle, 1982). The hydrophilicity profile is displayed in **Fig. 5.2**.

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CATTCAGAAAGTTTTCTTACAACTTGAATATTATTAAATAGTCAAATGCGCCTTTTTCTG

ATAAACTTAGGATGATGTTTTTTTGAAAGGATGAGCCCCTATGACACCAAATCGCGAAGA
                          RBS         M  T  P  N  R  E  D
CTATTTAAAATTAATTTTTGAATTAGGTGGCGACGAAGTTAAAGTGAATAATAAACAAAT
 Y  L  K  L  I  F  E  L  G  G  D  E  V  K  V  N  N  K  Q  I
TGTTTCTGGACTCGATGTTTCGGCAGCTTCGGTTAGTGAGATGATTTCAAAGTTAGTAAA
 V  S  G  L  D  V  S  A  A  S  V  S  E  N  I  S  K  L  V  K
AGAAGATTTGGTTGAGCATTCTCCTTATCAAGGGGTACAATTAACTGAAAAAGGCTTAAA
 E  D  L  V  E  H  S  P  Y  Q  G  V  Q  L  T  E  K  G  L  K
AAAAGCGAGTACGTTAATTCGCAAACACCGAATCTGGGAAGTCTTTTTAGTAGAGCACTT
 K  A  S  T  L  I  R  K  J  R  I  W  E  V  F  L  V  E  H  L
AAATTACACTTGGAATGATGTGCACGAAGAGGCAGAAGTTTTAGAACATGTTACTTCACA
 N  Y  T  W  N  D  V  H  E  E  A  E  V  L  E  H  V  T  S  Q
GACGCTTGTGAACCGTTTAGCGGATTATTTAAATCATCCAGAATTTTGTCCACACGGTGG
 T  L  V  N  R  L  A  D  Y  L  N  H  P  E  F  C  P  H  G  G
TGTTATTCCCGAAGATAATCAACCCATTCATGAGGAGAAACGCCAAACGTTAACAGACTA
 V  I  P  E  D  N  Q  P  I  H  E  E  K  R  Q  T  L  T  D  Y
CCCTGTTGGCACAAAAATTCGGATTGCACGTGTCTTAGACGAAAAAGAATTACTGGATTA
 P  V  G  T  K  I  R  I  A  R  V  L  D  E  K  E  L  L  D  Y
TTTAGTTTCCATTGATTTAAATATTCAAGAAGAATATACGATTAAAGAAATTGCTGCATA
 L  V  S  I  D  L  N  I  Q  E  E  Y  T  I  K  E  I  A  A  Y
TGAAGGACCGATCACCATTTATAATGAAAACAAAGAATTATCCGTCAGCTTTAAAGCAGC
 E  G  P  I  T  I  Y  N  E  N  K  E  L  S  V  S  F  K  A  A
AAACACAATTTTTGTTGAGCCGTTGATTAGAGAAAGTGAGGAAAACTAATGAATGAAAA
 N  T  I  F  V  E  P  L  I  R  E  S  E  E  N  *
```

Fig. 5.1. Nucleotide sequence of *E. faecalis* JH2-2 *efaR* as determined by sequencing. The inferred amino acid sequence is also included. The putative Ribosome Binding Site (RBS) is underlined.

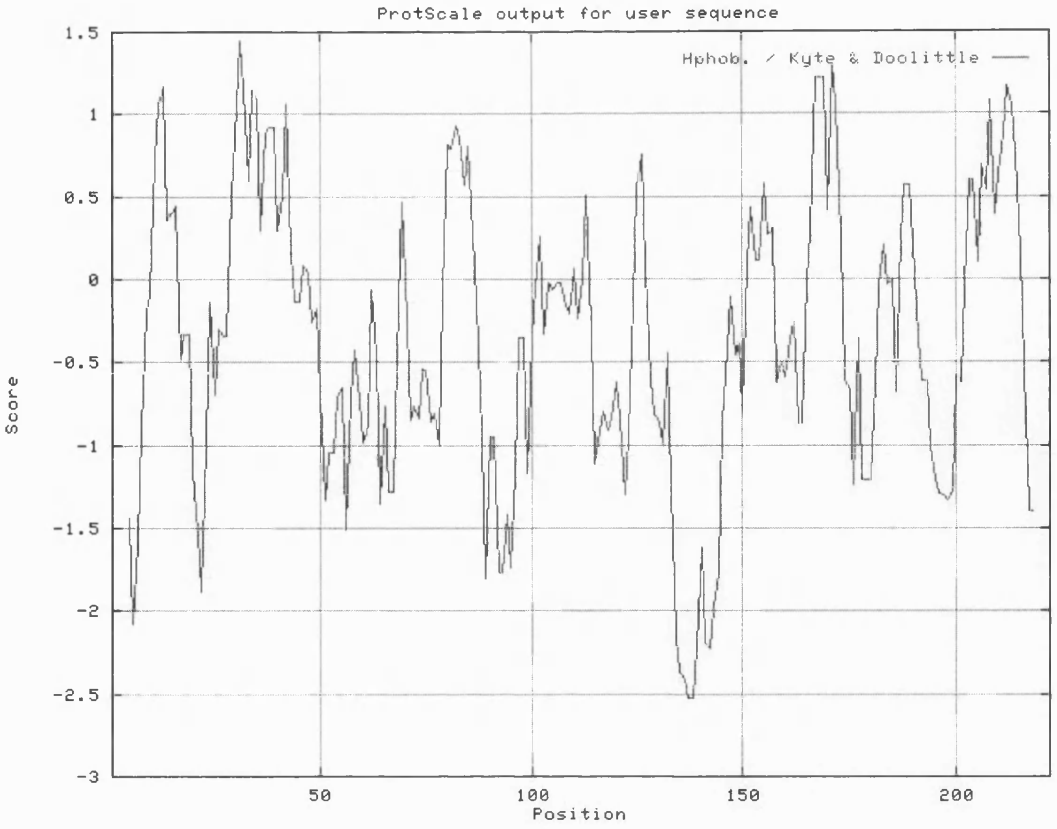


Fig. 5.2. Hydropathy profile of EfaR. This profile was generated using the ProtScale online programme available at the SWISS-PROT ExPASy website.

The DtxR protein of *C. diphtheriae*, encoded by the *dtxR* gene, is the archetype of the family of metalloregulatory proteins to which EfaR belongs. In corynebacteria, the *dtxR* gene immediately precedes *galE*, a gene which encodes the enzyme P-galactose 4-epimerase. The two genes have been shown to be co-transcribed in *Corynebacterium glutamicum* as bicistronic mRNA, particularly during the exponential growth phase, in addition to *dtxR* being transcribed as a monocistronic entity (Oguiza *et al.*, 1996). Examination of the 5000 bp flanking either side of *efaR* revealed only one significant ORF on the same strand. This ORF, lying immediately downstream of *efaR*, was homologous to the *zwf* gene of *Salmonella typhimurium*, which encodes the enzyme glucose-6-phosphate 1-dehydrogenase (G6PD). This

enzyme catalyses the first enzymatic step in the pentose phosphate pathway, which produces ribose for use in production of nucleosides and reducing equivalents in the form of NADPH. G6PD-deficient *S. typhimurium* were shown to have increased susceptibility to reactive oxygen and nitrogen intermediates, and their virulence in mice was found to be attenuated (Lundberg *et al.*, 1999). These findings were attributed to G6PD playing an important role in antioxidant defenses by maintaining the cellular redox state, regenerating reduced thiols, and repairing oxidative damage. NADPH is the electron source for several reductases that repair oxidative damage and regenerate antioxidant species, including glutathione reductase, thioredoxin reductase, and methionine sulphoxide reductase. The proximity of the *zwf* and *efaR* genes (separated as they are by a mere 2 bp) on the same strand, the bicistronic transcription of the similarly-organised *C. glutamicum dtxR-galE* genes, and the fact that the consensus binding sequence for SoxS binding (or sox box) as determined in *E. coli* by Griffith and Wolf (Griffith and Wolf, 2001) is not readily identifiable in the region of DNA that would constitute the putative *zwf* promoter strongly suggest the possibility that the two genes are co-transcribed. Such a prospect is most intriguing, bearing in mind the role of G6PD in antioxidant defence and the possible role(s) of the genes in the EfaR regulon in oxidative stress response (which will be discussed later in the chapter).

The primary and secondary structures of EfaR were analysed using a variety of freely available online facilities, e.g. GeneQuiz system available via the website of the European Molecular Biology Laboratory (EMBL, http://www.embl.org/). EfaR is a member of a relatively newly-discovered class of metal-responsive transcriptional regulators, the archetype of which is *C. diphtheriae* DtxR. BLAST

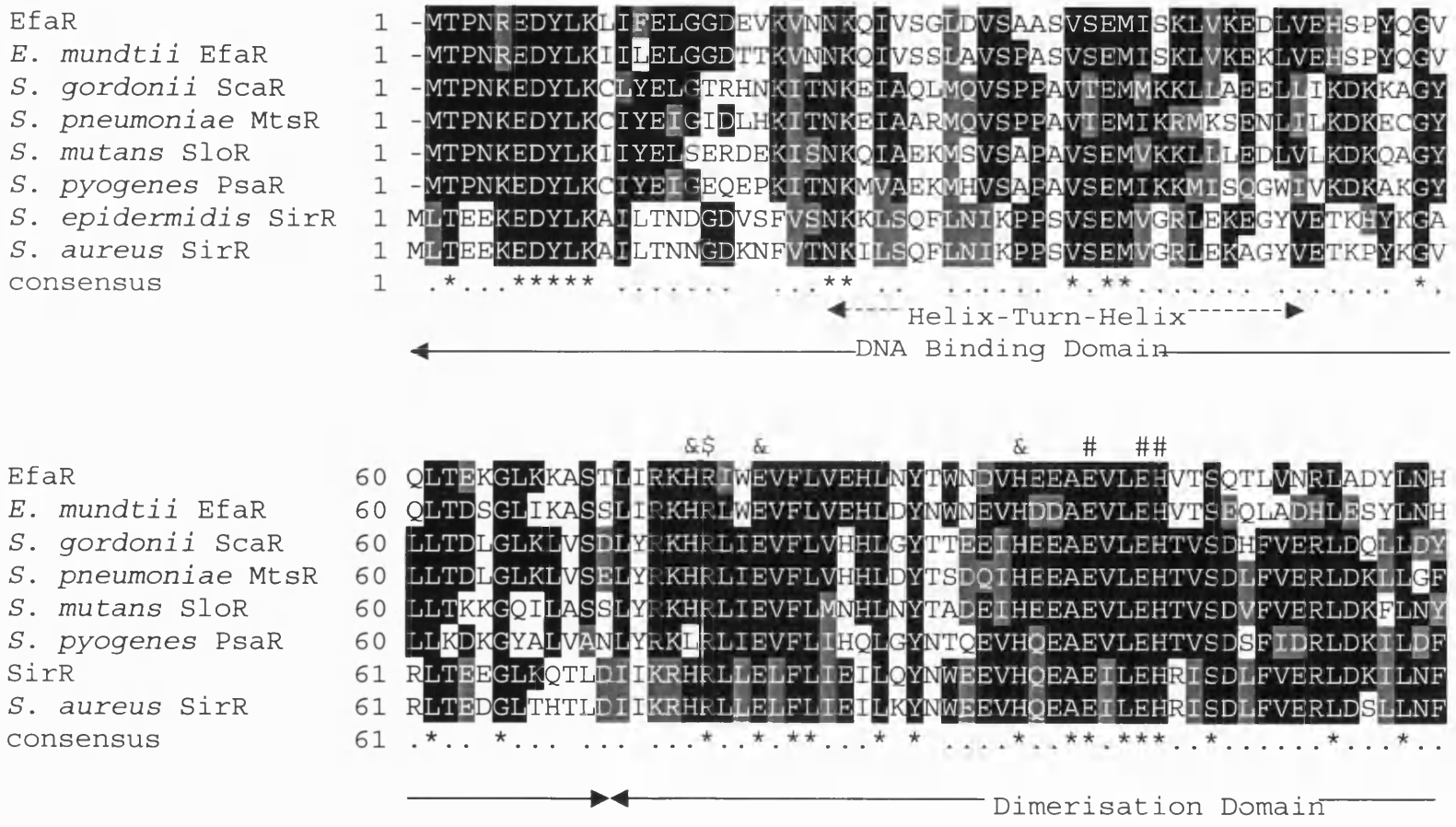
analysis revealed that the primary sequence of EfaR is 27% identical (45% similarity) to that of *C. diphtheriae* DtxR, 39% identical (56% similarity) to *S. epidermidis* SirR, 40% identical (57% similarity) to *S. pneumoniae* PsaR and 39% identical (56% similarity) to *S. gordonii* ScaR. The number of putative and known proteins in the Genbank and EMBL databases with which EfaR is homologous has expanded dramatically in the last few years and is continuing to grow, with homologues reported in microorganisms ranging from *Archaeoglobus* spp. to *Streptomyces* spp. A selection of homologues is presented in **Table 5.1**. Identity and similarity percentages were obtained by pair-wise comparison of amino acid residues against that of EfaR using the BLASTP facility provided on the NCBI website.

Sequence alignment was undertaken to analyse the conservation of individual residues in EfaR by comparison of its primary sequence with those of other Genbank and EMBL sequences, using the multiple sequence alignment programme ClustalW, which is freely available on the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/). For clarity, only selected sequences are presented in **Fig. 5.3** in order that domains and key features may be pointed out. A more comprehensive alignment comprising 24 sequences is featured in **Fig. 5.4**.

Table 5.1. A selection of known or putative proteins in the Genbank and EMBL databases homologous to EfaR.

Name	Bacterium	Identity (%)	Similarity (%)	Length (aa)	Accession number	Metal Ion(s)	Evidence of metal-responsiveness
(EfaR)	*Enterococcus mundtii*	75	86	215	AF417113	Mn?	
ScaR	*Streptococcus gordonii*	39	56	215	AF182402	Mn	Jakubovics *et al.*, 2000
SloR	*Streptococcus mutans*	46	62	217	AF232688	Mn?/Fe	Spatafora *et al.*, 2001
MtsR	*Streptococcus pneumoniae* R6	40	57	216	AE008517	Mn?	
(PsaR)	*Streptococcus pyogenes* M1 GAS	40	57	215	AE006505	Mn?	
SirR	*Staphylococcus epidermidis*	39	56	214	X99128	Mn/Fe	Hill *et al.*, 1998
(SirR)	*Staphylococcus aureus*	42	57	214	AF452622	Mn?/Fe?	
	Lactococcus lactis subsp. *lactis*	35	54	217	AE006357	Mn?/Fe?	
TroR	*Treponema pallidum*	34	52	153	AE001201	Mn	Posey *et al.*, 1998
SirR	*Mycobacterium tuberculosis*	36	53	228	AL008967	Mn?/Fe?	
	Deinococcus radiodurans R1	37	55	232	AAF12080	Mn?/Fe?	
SirR	*Halobacterium* sp. NRC-1	34	49	233	AE005005	Mn?/Fe?	
MntR	*Bacillus subtilis*	28	53	142	P54512	Mn	Que & Helmann, 2000
(MntR)	*Bacillus halodurans*	31	55	139	AP001516	Mn?	
MntR	*Escherichia coli* K12	32	55	155	A64819	Mn	Patzer & Hantke, 2001
	Methanothermobacter thermautotrophicus	25	44	241	AE000808	Mn?/Fe?	
DtxR	*Corynebacterium diphtheriae*	27	45	226	P33120	Fe	Boyd *et al.*, 1990
DtxR	*Corynebacterium glutamicum*	27	49	228	L35906	Fe	Oguiza *et al.*, 1995
IdeR	*Rhodococcus equi*	32	53	230	AF277002	Fe	Boland & Meijer, 2000
DtxR	*Rhodococcus erythropolis*	32	53	228	AF277296	Fe?	
IdeR	*Mycobacterium tuberculosis*	36	56	230	AE007107	Fe	Schmitt *et al.*, 1995
IdeR	*Mycobacterium leprae*	34	55	230	AL583920	Fe?	
	Streptomyces lividans	28	47	230	Z50049	Fe?	

To get a better idea of the relatedness of the various EfaR homologues, a dendrogram was generated using data obtained from ClustalW analysis of EfaR homologues. From the resultant plot (**Fig. 5.5**) it is apparent that the proteins can generally be divided into two families. One family consists mainly of enterococcal, streptococcal and staphylococcal proteins homologous to EfaR, ScaR and SirR. Some members of this family have been characterised as being either Mn^{2+}-responsive, or responsive to both Mn^{2+} and Fe^{2+}. The second family is more diverse, containing proteins homologous to *B. subtilis* MntR, *C. diphtheriae* DtxR and *M. tuberculosis* IdeR. As DtxR and IdeR are known to be Fe^{2+}-responsive *in vivo*, it is likely that their homologues will also be Fe^{2+}-responsive. In contrast, the MntR-like homologues will more likely be Mn^{2+}-responsive. This family also contains proteins whose overall homologies places them in this family but whose sequences at key motifs are more akin to those of the first family, e.g. the metal-binding sites of the *M. tuberculosis* SirR protein resemble that of *S. epidermidis* SirR more closely that it does those of *C. diphtheriae* DtxR. It is hence tempting to speculate that *M. tuberculosis* SirR binds Mn^{2+}. Curiously, a trawl though the firmicute genomes on the TIGR site revealed that only the mycobacteria possessed two DtxR homologues in their genomes. Why this should be the case can only be speculated about. It is suspected the same applies to the corynebacteria and rhodococci, but their genomes were not available for searching through TIGR.


EfaR
E. mundtii EfaR
S. gordonii ScaR
S. pneumoniae MtsR
S. mutans SloR
S. pyogenes PsaR
S. epidermidis SirR
S. aureus SirR
consensus
Helix-Turn-Helix
DNA Binding Domain
Dimerisation Domain

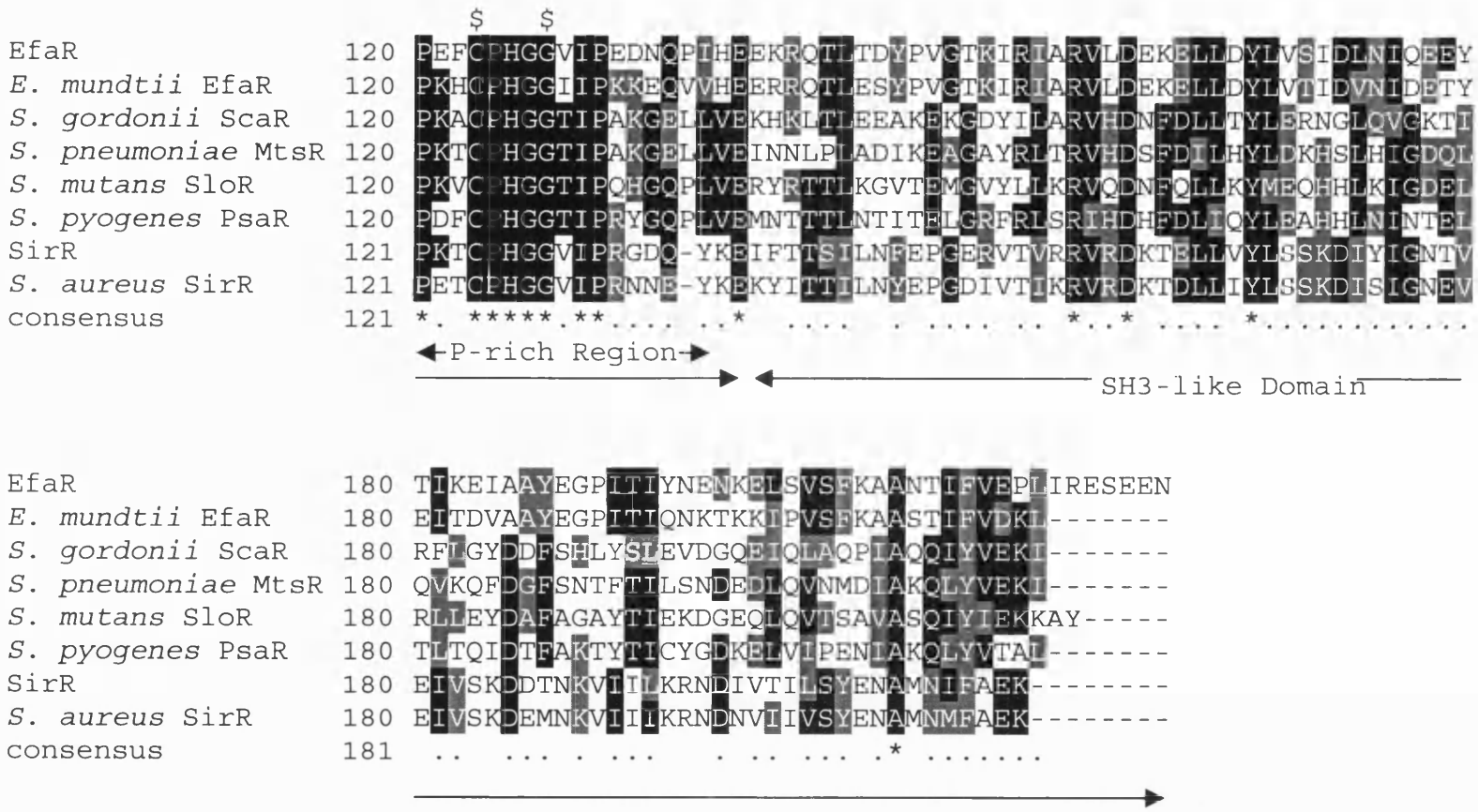
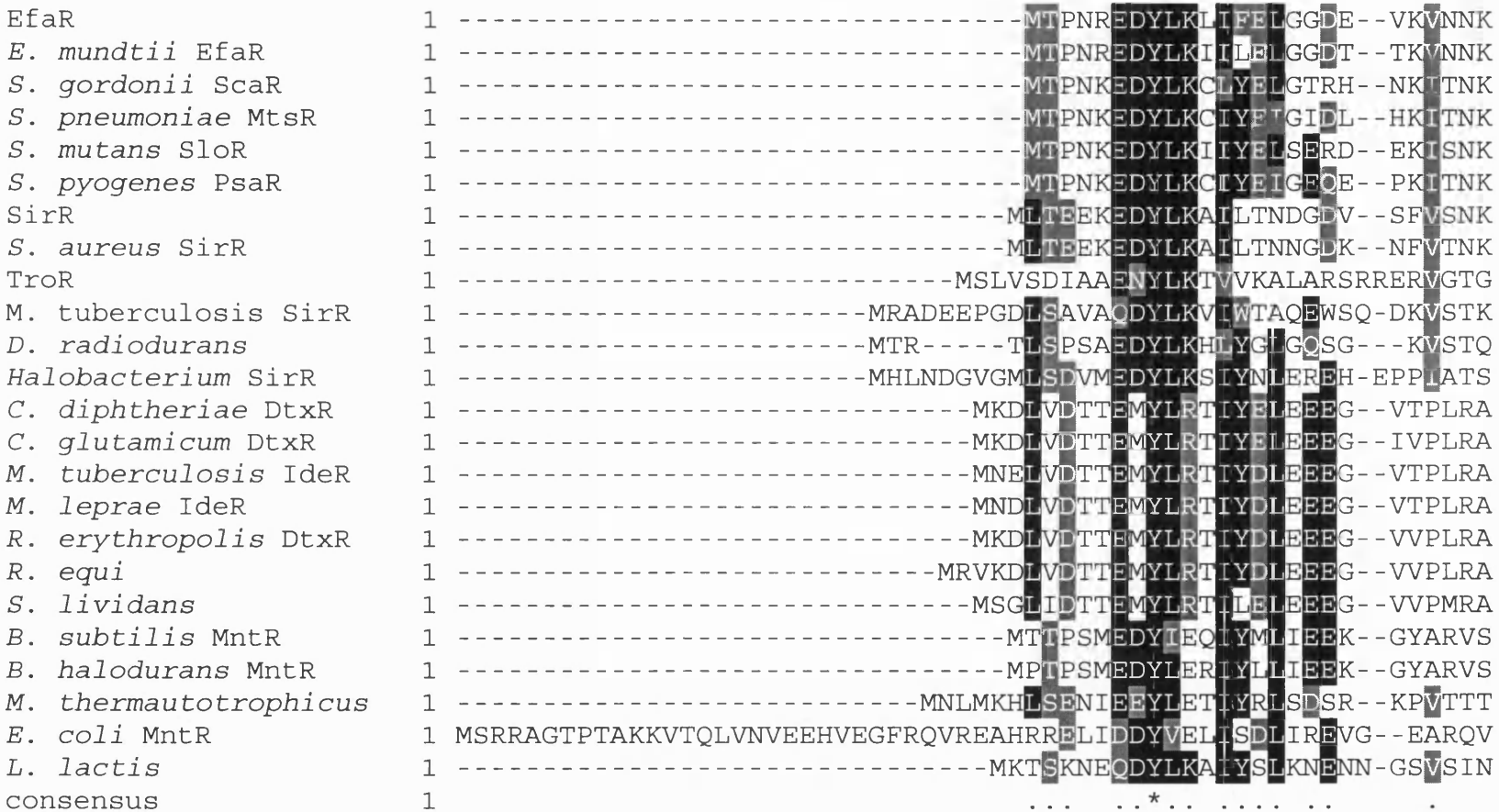


Fig. 5.3. Box-shaded ClustalW analysis of EfaR and its streptococcal, staphylococcal and enterococcal homologues. Dashes represent gaps introduced to optimise alignments. Other symbols denote features as follows: &, metal binding site 1; #, metal binding site 2; $, anion-binding sites; . in the consensus line, conserved residues; *, identical residues.


EfaR 1 ----------MTPNREDYLKLIFELGGDE--VKVNNK
E. mundtii EfaR 1 ----------MTPNREDYLKIILELGGDT--TKVNNK
S. gordonii ScaR 1 ----------MTPNKEDYLKCLYELGTRH--NKITNK
S. pneumoniae MtsR 1 ----------MTPNKEDYLKCIYEIGIDL--HKITNK
S. mutans SloR 1 ----------MTPNKEDYLKIIYELSERD--EKISNK
S. pyogenes PsaR 1 ----------MTPNKEDYLKCIYEIGEQE--PKITNK
SirR 1 ----------MLTEEKEDYLKAILTNDGDV--SFVSNK
S. aureus SirR 1 ----------MLTEEKEDYLKAILTNNGDK--NFVTNK
TroR 1 ----------MSLVSDIAAENYLKTVVKALARSRRERVGTG
M. tuberculosis SirR 1 ----------MRADEEPGDLSAVAQDYLKVIWTAQEWSQ-DKVSTK
D. radiodurans 1 ----------MTR-----TLSPSAEDYLKHLYGLGQSG---KVSTQ
Halobacterium SirR 1 ----------MHLNDGVGMLSDVMEDYLKSIYNLEREH-EPPLATS
C. diphtheriae DtxR 1 ----------MKDLVDTTEMYLRTIYELEEEG--VTPLRA
C. glutamicum DtxR 1 ----------MKDLVDTTEMYLRTIYELEEEG--IVPLRA
M. tuberculosis IdeR 1 ----------MNELVDTTEMYLRTIYDLEEEG--VTPLRA
M. leprae IdeR 1 ----------MNDLVDTTEMYLRTIYDLEEEG--VTPLRA
R. erythropolis DtxR 1 ----------MKDLVDTTEMYLRTIYDLEEEG--VVPLRA
R. equi 1 ----------MRVKDLVDTTEMYLRTIYDLEEEG--VVPLRA
S. lividans 1 ----------MSGLIDTTEMYLRTILELEEEG--VVPMRA
B. subtilis MntR 1 ----------MTTPSMEDYIEQIYMLIEEK--GYARVS
B. halodurans MntR 1 ----------MPTPSMEDYLERIYLLIEEK--GYARVS
M. thermautotrophicus 1 ----------MNLMKHLSENIEEYLETIYRLSDSR--KPVTTT
E. coli MntR 1 MSRRAGTPTAKKVTQLVNVEEHVEGFRQVREAHRRELIDDYVELISDLIREVG--EARQV
L. lactis 1 ----------MKTSKNEQDYLKAIYSLKNENN-GSVSIN
consensus 1 ... ..*.. ....

EfaR 26
E. mundtii EfaR 26
S. gordonii ScaR 26
S. pneumoniae MtsR 26
S. mutans SloR 26
S. pyogenes PsaR 26
SirR 27
S. aureus SirR 27
TroR 32
M. tuberculosis SirR 36
D. radiodurans 29
Halobacterium SirR 36
C. diphtheriae DtxR 29
C. glutamicum DtxR 29
M. tuberculosis IdeR 29
M. leprae IdeR 29
R. erythropolis DtxR 29
R. equi 31
S. lividans 29
B. subtilis MntR 27
B. halodurans MntR 27
M. thermautotrophicus 32
E. coli MntR 59
L. lactis 29
consensus 61

```
EfaR                          86 HLNYTWNDVHEEAEVLEHVISQTLVNRLADYLNHPEFCPHGGVIPE--------------
E. mundtii EfaR               86 HLDYNWNEVHDDAEVLEHVTSEQLADHLESYLNHPKHCPHGGIIPK--------------
S. gordonii ScaR              86 HLGYTTEEIHEEAEVLEHTVSDHFVERLDQLLDYPKACPHGGTIPA--------------
S. pneumoniae MtsR            86 HLDYTSDQIHEEAEVLEHTVSDLFVERLDKLLGFPKTCPHGGTIPA--------------
S. mutans SloR                86 HLNYTADEIHEEAEVLEHTVSDVFVERLDKFLNYPKVCPHGGTIPQ--------------
S. pyogenes PsaR              86 QLGYNTQEVHQEAEVLEHTVSDSFIDRLDKILDFPDFCPHGGTIPR--------------
SirR                          87 ILQYNWEEVHQEAEILEHRISDLFVERLDKILNFPKTCPHGGVIPR--------------
S. aureus SirR                87 ILKYNWEEVHQEAEILEHRISDLFVERLDSLLNFPETCPHGGVIPR--------------
TroR                          92 VLCLEAGVVHKEAEMLEHACSDELIDVIDRYLQYPTRDPHGQPIPR--------------
M. tuberculosis SirR          96 ELGYRWDEVHDEAEVLEHAVSDRLMARIDAKLGFPQRDPHGDPIPG--------------
D. radiodurans                89 ALGVPLDEVHDEAEALEHALSDRLEARIAAWLGDPTHDPHGDPIPT--------------
Halobacterium SirR            96 HLDYDWDEVHDEADALEHHISEEFEDRLAEKLGDPTVDPHGDPIPN--------------
C. diphtheriae DtxR           89 IIGLDINKVHDEACRWEHVMSDEVERRLVKVLKDVSRSPFGNPIPGLDELG------VGN
C. glutamicum DtxR            89 IIGLDIHKVHDEACRWEHVMSLEVERRLVEVLDDVHRSPFGNPIPGLGEIG------LDQ
M. tuberculosis IdeR          89 VIGLPWEEVHAEACRWEHVMSEDVERRLVKVLNNPTTSPFGNPIPGLVELG------VGP
M. leprae IdeR                89 VIGLPWEEVHAEACRWEHVMSEDVERRLIKVLNNPTTSPFGNPIPGLLDLG------AGP
R. erythropolis DtxR          89 VIGLKWEDVHAEACRWEHVMSEEVERRLVVVLNNPTTSPYGNPIPGLDQLG------LDG
R. equi                       91 IIGLEWDQVHAEACRWEHVMSEDVERRLVEVLKNPTTSPYGNPIPGLADLG------LDR
S. lividans                   89 VIGLEWEQVHAEACRWEHVMSEAVERRVLELLRHPTESPYGNPIPGLEELGE-----TDG
B. subtilis MntR              86 IIGVDEEKIYNDVEGIEHHLSWNSIDRIGDLVQY--------------------------
B. halodurans MntR            86 MIGVDSDHIYEDVEGIEHHLSWDAIDRIGDLVQY--------------------------
M. thermautotrophicus         92 VLGIKRERIHRQACEMEHSLSDDAERALCHLLNMPGECPDEKPIPACEFKFQTCEECIEM
E. coli MntR                 118 VLGVSPEIARRDAEGMEHHVSEETLDAFRLFTQKHGAK----------------------
L. lactis                     89 KVGYTMEEVHDEAENLEHASSPRLIESLYVLMGYPATDPHGSEIPT--------------
consensus                    121 .... ........ .** .*. ..... ... .  ... ...
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EfaR 132 ----DNQPIHEEKR-QTLTDYPVGTKIRIARVLD-EKELLDYLVSIDLNIQEEYTIKEIA
E. mundtii EfaR 132 ----KEQVVHEERR-QTLESYPVGTKIRIARVLD-EKELLDYLVTIDVNIDETYEITDVA
S. gordonii ScaR 132 ----KGELLVEKHK-LTLEEAKEKGDYILARVHD-NFDLLTYLERNGLQVGKTIRFLGYD
S. pneumoniae MtsR 132 ----KGELLVEINN-LPLADIKEAGAYRLTRVHD-SFDILHYLDKHSLHIGDQLQVKQFD
S. mutans SloR 132 ----HGQPLVERYR-TTLKGVTEMGVYLLKRVQD-NFQLLKYMEQHHLKIGDELRLLEYD
S. pyogenes PsaR 132 ----YGQPLVEMNT-TTLNTITELGRFRLSRIHD-HFDLIQYLEAHHLNINTELTLTQID
SirR 133 ----GDQ-YKEIFT-TSILNFEPGERVTVRRVRD-KTELLVYLSSKDIYIGNTVEIVSKD
S. aureus SirR 133 ----NNE-YKEKYI-TTILNYEPGDIVTIKRVRD-KTDLLIYLSSKDISIGNEVEIVSKD
TroR 138 ----KDTLLDLYVE-DDVPGV----------
M. tuberculosis SirR 142 ----ADGQVPTPPA-RQLWACRDGDTGTVARISDADPQMLRYFASIGISLDSRLRVLARR
D. radiodurans 135 ----LEGELPARAE-RRLSQLAPGDHAVIARVPDGDAEQLRTLVGARLTPGARLQIRSVD
Halobacterium SirR 142 ----ADLEPPEHPDTEVLADHAEGDALVVARASLRNTDELRYLADAGVQPGTELTVTEHA
C. diphtheriae DtxR 143 SDAAAPG--TRVIDAATSMP-RKVRIVQINEIFQVETDQFTQLLDADIRVGSEVEIVDRD
C. glutamicum DtxR 143 ADEPDSG--VRAIDLPLGEN-LKARIVQLNEILQVDLEQFQALTDAGVEIGTEVDIINEQ
M. tuberculosis IdeR 143 EPGADDANLVRLTELPAGSP-VAVVVRQLTEHVQGDIDLITRLKDAGVVPNARVTVETTP
M. leprae IdeR 143 DASAANAKLVRLTELPSGSP-VAVVVRQLTEHVQDDIDLITRLKDTGVVPNARVTVETSP
R. erythropolis DtxR 143 -PVATTETLVRLTDIPHGAQ-TAVVVRRLAEHVQSDPEVIAQLRDAGVVPDARVTVETRP
R. equi 145 -PVGNAETLIRLTDVPPGKP-TAVVVRRLAEHVQSDPELIGQLREAGVVPDARVTVETRP
S. lividans 144 ADPFLDEGMVSLADLDPGQEGKTVVVRRIGEPIQTDAQLMYTLRRAGVQPGSVVSVTESA
B. subtilis MntR 120 ----FEEDDARKKDLKSIQK-KTEHHNQ----------
B. halodurans MntR 120 ----FQEDPSRLNDLREVQK-KNEE----------
M. thermautotrophicus 152 KDADIEEIGCRDENLKSLTE-MDENQSGRVSFIRGDYRVVRRLMDMGITTIGAPLTMIKRA
E. coli MntR ----------
L. lactis 135 -----ESFWLKNEVELTLDEAQVEQTYHVTLIEE---EGKNFFKKLEISLPHLIKVIDIL
consensus 181

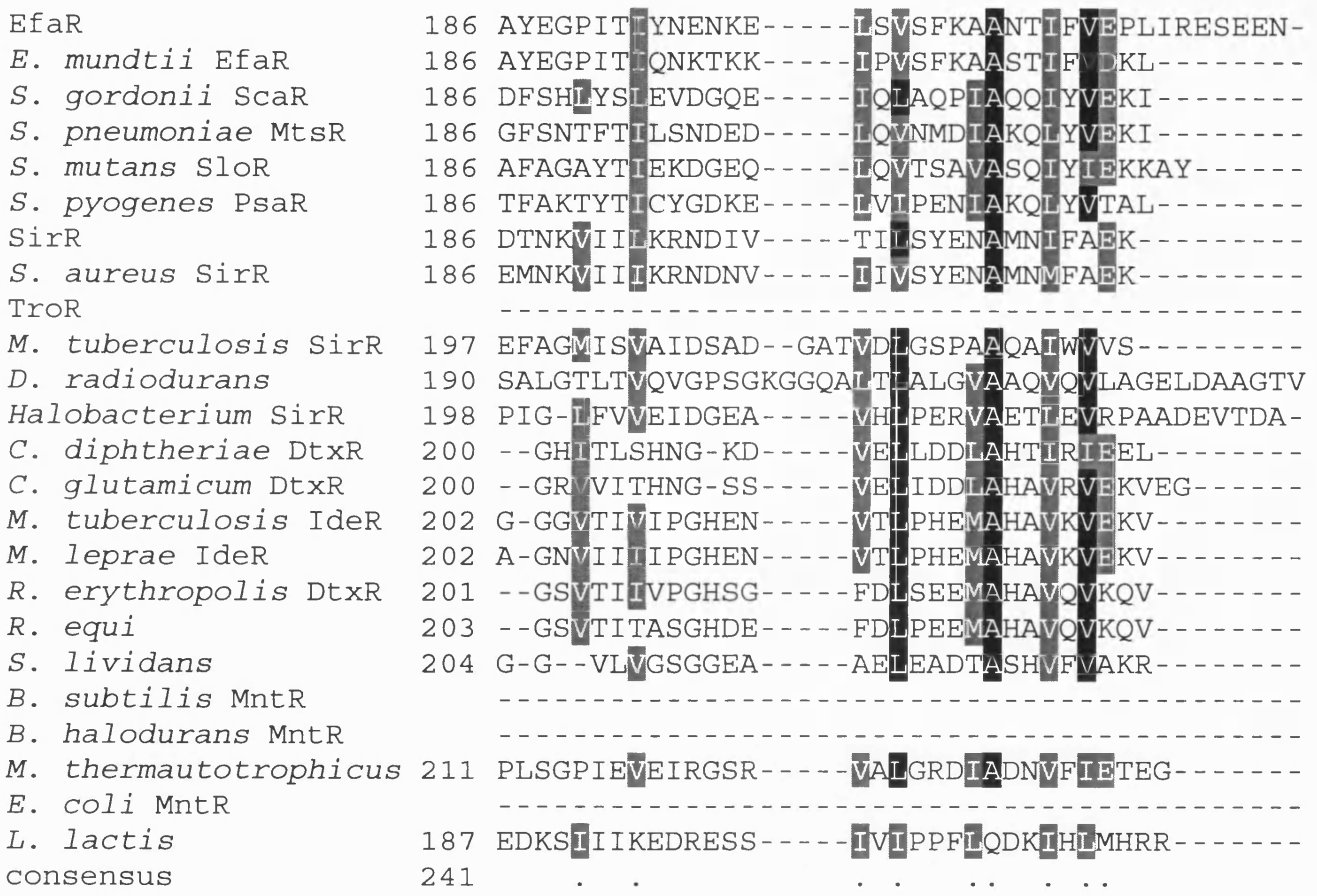

Fig. 5.4. Box-shaded ClustalW analysis of EfaR and twenty three homologues. Dashes represent gaps introduced to optimise alignments. Other symbols denote features as follows: (.), conserved residues; (*), identical residues.

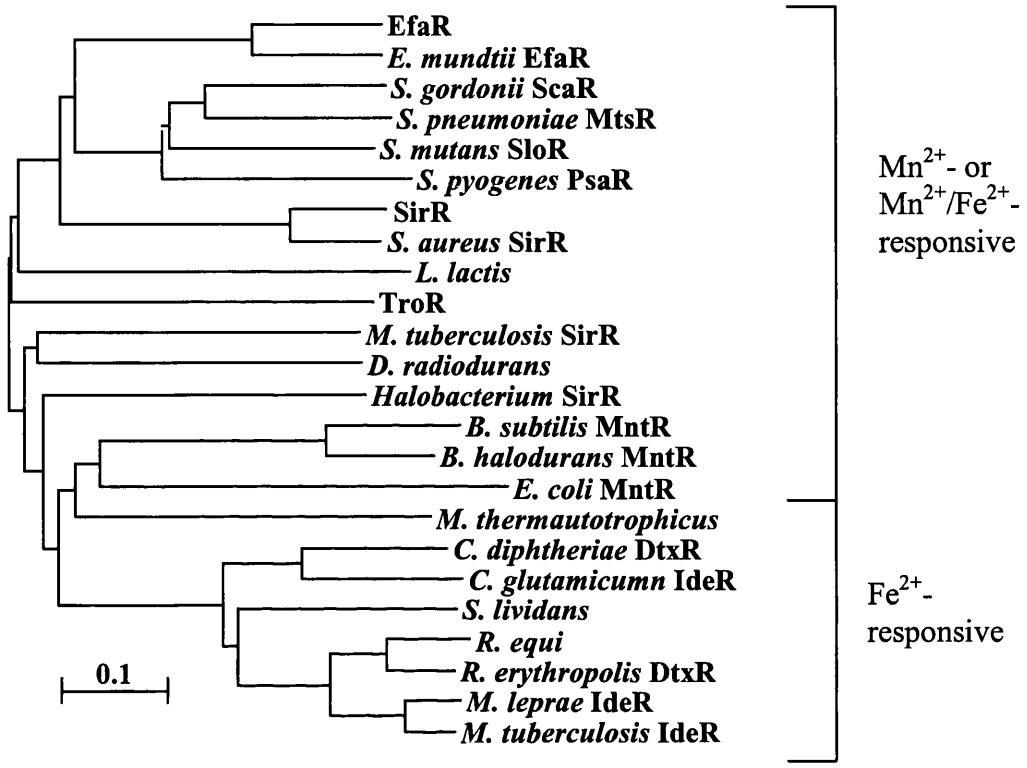

Fig. 5.5. Dendrogram generated using data obtained from ClustalW analysis of EfaR homologues. The proteins fall into one of two major families, which can be further divided into subclusters generally along genus lines. The metal ion assignations shown above are on the basis of experimental evidence where available, or on the basis of sequence homology.

Analysis of the major domains reveals that the DNA binding domains of the corynebacteria, mycobacteria (DtxR-type homologues, not SirR-type), rhodococci and streptomyces form a clearly-defined block. In contrast, the DNA binding domains of the other proteins are rather more diverse, with a high degree of intra-genus residue conservation and a lesser degree between genera. Despite the similarity of the EfaR and SirR binding sequences, the staphylococcal and enterococcal sequences did not display as great a degree of homology as might have been expected. A greater degree of similarity can be discerned in the dimerisation domain, it being possible to define three major groups (with some minor sub-groups on genus lines). The first includes the streptococcal, enterococcal, staphylococcal and mycobacterial SirR-like homologues, the second comprises the *B. subtilis* and *E. coli* MntR-like proteins and the third group encompasses the mycobacterial DtxR-like proteins, corynebacteria and rhodococci. The SH3-like domain exhibits the greatest degree of sequence divergence amongst the three domains. Indeed, this domain is even absent from the MntR-like proteins. Unlike the second domain, major groups cannot easily be defined, and sequence conservation is really only obvious intra-genera.

Two homologues, DtxR and *M. tuberculosis* IdeR, have been crystallised and their structures comprehensively analysed (Pohl *et al.*, 1999a; Pohl *et al.*, 1999b; Qiu *et al.*, 1996). It was such crystallographic studies that originally suggested that there are three major domains (**Fig. 5.3**). These are an N-terminal DNA-binding domain containing a canonical prokaryotic helix-turn-helix (HTH) motif, a central α-helical dimerisation domain containing metal ion co-ordinating residues, and a C-terminal SH3-like domain thought to interact with the metal-binding and DNA-binding

domains (Pohl *et al.*, 1999b). It is thought that the apo-proteins exist in solution as inactive loosely-folded monomers incapable of DNA binding. Solution NMR structural studies and fluorescence binding assays of apo-DtxR suggest that in solution, the first two domains are loosely-folded, conformationally flexible and hence lacking in tertiary structure, whereas the third domain is well-ordered (Twigg *et al.*, 2001). The lack of differences in conformational behaviour between the first two domains has led one group to suggest that those domains might be considered as one and the same (Twigg *et al.*, 2001). Binding of divalent metal cations induces a conformational change in the loosely-folded DNA-binding and dimerisation domains resulting in a more ordered state, which has been termed a "disorder to order" folding transition (Twigg *et al.*, 2001). This metal-activated reorganisation stabilises the dimeric complex (Tao *et al.*, 1995) thus facilitating repressor function. Two dimeric holorepressor molecules bind simultaneously to an operator sequence on opposite faces of the DNA helix (White *et al.*, 1998). As a result, the DNA helical axis becomes distorted slightly compared with that of linear canonical B-form DNA (Pohl *et al.*, 1999b), enabling the recognition helix of the helix-turn-helix motif from each monomer to insert into the major groove.

Crystallographic studies revealed the presence of two distinct metal binding sites in DtxR (Ding *et al.*, 1996; Schiering *et al.*, 1995; Qiu *et al.*, 1995), yet binding assays suggested only one high affinity site per monomer (Wang *et al.*, 1994). Site-directed mutagenesis studies eventually revealed that metal binding site 2 (C102, E105, H106) was essential to repressor activity whereas metal binding site 1 (H79, E83, H98) was merely an ancillary site that mattered little, if at all, (Ding *et al.*, 1996). In contrast, the anion binding residues (R80, S126 and N130) associated with metal

binding site 1 were essential for DtxR activity (Goranson-Siekierke *et al.*, 1999), possibly by assisting in the ordering of domains or in dimer stabilisation.

Although there is a high degree of conservation amongst the metal ion-binding residues in particular and the dimerisation domain in general, the various DtxR homologues have different and specific metal ion preferences *in vivo*, although most can bind a wide range of metal cations *in vitro*. Several of the DtxR-like subgroup, e.g. the *C. diphtheriae* DtxR and *M. tuberculosis* IdeR proteins, have been reported as being specific *in vivo* for Fe^{2+}. In contrast, the *B. subtilis* MntR and *S. gordonii* ScaR homologues are Mn^{2+}-specific *in vivo* (Que and Helmann, 2000; Jakubovics *et al.*, 2000). Curiously, the ScaR-like protein SloR has been reported to be Fe^{2+}-specific *in vivo* (Spatafora *et al.*, 2001). In common with its homologues, EfaR bound a range of metal ions *in vitro*, but the fact that EfaA expression and *efaCBA* transcript production were sensitive only to Mn^{2+} (**Chapter 4**) suggests that EfaR may be Mn^{2+}-specific *in vivo*.

A possible explanation for the differences in cation specificity between homologues lies in the mechanism of dimer formation. Wang *et al.* (Wang *et al.*, 1999) proposed a model for dimerisation in which the proline-containing region (S126-G139) that bridges the dimerisation and SH3-like domains serves as an internal molecular switch for dimer formation. In their model, binding of divalent cations causes this region to be associated with the N-terminal domain, stabilising the active metal-containing dimeric holorepressor. Alternatively, in the absence of bound metal the proline-rich region associates with the C-terminal SH3-like domain in a manner reminiscent of the binding by eukaryotic SH3 domains of proline-containing

peptides, stabilising the inactive repressor monomer instead. Such an explanation is consistent with findings that C-terminal domain mutants are constitutively active even in the absence of iron (Sun *et al.*, 1998), and that mutations of R80, S126 and N130 (the anion-binding residues) severely decreased DtxR activity (Goranson-Siekierke *et al.*, 1999). The latter was interpreted by Wang *et al.* as the result of disruption of binding between the proline-rich region and the N-terminal domain (Wang *et al.*, 1999). In spite of having been identified as critical for DtxR functionality, S126 and N130 have in the staphylococcal, enterococcal and streptococcal homologues been substituted by other residues (S126C and N130G). Furthermore, whereas the DtxR residues G134-G141 in that proline-rich region are also highly conserved in IdeR, other residues are conserved in this region amongst the other genera, with the streptococcal proteins seeming to form a separate sub-cluster. Finally, the coordinating residues of the metal and anion binding sites of EfaR, SirR, ScaR, SloR and MtsR are fully conserved amongst these proteins, but comparison with DtxR and IdeR reveals that the former group differ from the latter by conservative substitutions at metal binding site 2 (C102E) as well as the aforementioned differences in anion binding site 1, although Tao and Murphy found that DtxR activity was abolished by all possible amino acid substitutions aside from aspartate (Tao and Murphy, 1993). Hence, the apparent variations in cation specificity between DtxR, EfaR and other homologues could be attributable to differences in substrate binding ligands resulting in differences in interactions with the SH3-like domain. It should be noted however that the absence of SH3-like C-terminal domains in homologues such as those from *T. pallidum* (TroR) and *B. subtilis* (MntR) (Posey *et al.*, 1999; Que and Helmann, 2000) implies that the switch theory is not necessarily universal and that cation selection may occur

somewhat differently in these proteins. More plausibly, it could well be that the proline switch theory represents a redundant inhibitory mechanism, and that the "disorder-to-order" structural transition enabled or imposed by cation-binding is sufficient for activation.

Taking the above into account, a slightly different hypothesis is proposed to explain the apparent variations in cation specificity *in vivo* and *in vitro* of DtxR, ScaR, EfaR and other homologues. It is proposed that in solution (as opposed to in crystals), differences in substrate binding ligands and their precise geometric arrangement in the metal binding sites due to differences in primary structure leads to the formation of structural variants. A range of cations might possess suitable ionic radii for the triggering of the disorder-to-order transition in a given homologue. However, depending on the primary sequence of the homologue, only one or two of the various cations possess radii capable of resulting in optimal folding, the rest resulting in less-than-ideal structural variants. Compared with the optimally-structured repressor, the structural variants could possess relatively low affinity for the target DNA sequence, adversely affecting the length of time for which they remain bound. The binding of repressor proteins to DNA is usually a dynamic process, and *in vitro*, repeated rebinding to DNA could well mask differences between the various metal-associated variants, resulting in an apparently wide cation-specificity. In other words, a protein repeatedly rebinding to a piece of DNA might retard the progression of the DNA to a similar degree to another protein which stayed bound longer. *In vivo*, however, the necessity of having to compete against RNA polymerase would place these on-optimal variants at a significant disadvantage. Because the optimally-folded repressors would "sit" longer on the

DNA, they would be able to block RNA polymerase access more effectively, hence the far stricter cation specificity *in vivo*. Alternatively, rather than DNA binding, it could be that structural variants form less stable dimers (holorepressor molecules), but a similar end result would ensue.

As mentioned in **Chapter 4**, the EfaR footprints on *efaCp* (approximately 21 bp) appeared to be smaller than those reported for ScaR with *scaCp* (approximately 46 bp). The differences were attributed to differences in the conservation of the binding sequences, with that in *E. faecalis* being considerably closer to the DtxR-like binding sequence consensus than that of the streptococci. Additionally, it has been hypothesised that EfaR could not bind *scaCp* because ScaR (but not EfaR) was able to bind to a separate highly conserved AT-rich palindrome present only in the streptococcal permease promoters rather than, or in addition to, the DtxR-like binding motif. Indeed, a previous study found that the streptococcal proteins form a closely-related subcluster from which EfaR can be excluded (*S. pyogenes* MtsR and *S. pneumoniae* PsaR are 53% and 64% identical to ScaR, respectively, compared with 39% for EfaR) (Jakubovics *et al.*, 2000). This makes it somewhat paradoxical therefore that EfaR should share more homology with members of the streptococcal sub-cluster than it does with other DtxR-like proteins like SirR and DtxR. However, examination of the residues comprising the helix-turn-helix motives of the two enterococcal, four streptococcal and two staphylococcal homologues (**Fig. 5.3**) does reveal conserved substitutions in this key DNA-recognition motif along generic lines (**Table 5.2**). In other words, each genera could be said to form a sub-cluster of its own in terms of the DNA recognition element. Comparison within genera reveals a greater degree of sequence divergence between any two streptococcal homologues

than is the case between members of the other genera. It is tempting to speculate that the comparative lack of residue conservation in the last third of the HTH motifs between the enterococci and streptococci might account for the apparent inability of EfaR to bind the *S. gordonii scaC* promoter. Despite its absence in a few homologues, evidence that the C-terminal SH3-like domain can influence the orientation of the DNA-binding domain (Pohl *et al.*, 1999b) should neither be forgotten nor discarded. Since there is considerably less homology between the proteins in this region, and it is equally plausible that differences in this domain may prove to be the key.

Homologue	**Helix-Turn-Helix residues**
E. faecalis EfaR	NKQIVSGLDVSAASVSEMISKLVKE
E. mundtii	S A P
S. epidermidis SirR	NKKLSQFLNIKPPSVSEMVGRLEKE
S. aureus	I
S. gordonii ScaR	NK**E**I**A**Q**LMQ**V**S**PP**A**V**T**EM**MK**KL**LA**E
S. pneumoniae	**AR** **I** **RMKS**
S. mutans	**Q** **EK** **S** **A** S **V** L
S. pyogenes	**MV** **EK** **H** **A** S **I** **MISQ**
Consensus	NK ia lqvspp VsEMikklvke

Table 5.2. Comparison of residues comprising the helix-turn-helix motifs of EfaR-like proteins. First, the archetypal proteins from each genera (EfaR, SirR or ScaR) were compared. Residues in the latter two differing from those in EfaR are denoted by underscores. Residues in ScaR differing from those in SirR are denoted in bold-type. Next, the other proteins within a genera were compared to the appropriate archetype. Only residues differing from that of the archetype are displayed. Underscores and bold-type were added following comparison with EfaR and ScaR, respectively. In the consensus for all eight proteins, upper case letters denote identical residues while lower case letters reflect conserved residues.

Comparison of Known DtxR-Like Binding Sequences

As noted in **Chapter 4**, analysis of the nucleotide sequence of the putative *E. faecalis efaCBA* promoter revealed two interrupted palindromic sequences of dyad symmetry. These DNA sequences possessed a high degree of homology to the SirR binding sequence within the *S. epidermidis sit* operon and to the DtxR operator consensus. However, as with the *S. epidermidis sit* operator, the *E. faecalis* palindromes (Hill *et al.*, 1998) lack the central five bases of the DtxR operator consensus.

The 19 bp DtxR operator consensus (TTAGGTTAGCCTAACCTAA) had been derived from a comparison of six DtxR-regulated *C. diphtheriae* promoters (Lee *et al.*, 1997; Lee and Holmes, 2000). Our observation prompted us to reassess the *C. diphtheriae* sequences and it became clear that the IRP operators could also have been interpreted as 14 bp motifs (**Fig. 5.6A**). In fact, when the enterococcal and staphylococcal binding sites are considered, the alternative interpretation yields a better alignment with a higher degree of consensus (**Fig. 5.6B**). It also becomes clear that the 19 bp motif is the result of the right "arm" of the 14 bp (CCTAA) is repeated. Conservation of this additional right arm "repeat" is relatively high in *C. diphtheriae* and progressively degenerates through the staphylococci, streptococci and the enterococci (becoming totally unrecognisable in the last). In contrast, the right arm in the "original" position is best conserved in the enterococcal and staphylococcal operators, less well-conserved in *C. diphtheriae* sequences, and becoming unrecognisable in the streptococcal sequences. On the basis of these

realignments (and further alignments to be discussed later) a new 14 bp consensus is proposed: TTAGGNNNNCCTAA (where N = A, T, C or G).

The finding in **Chapter 4** that EfaR was unable to bind streptococcal sequence suggests that EfaR is unable to recognise right arm sequence in the repeat position, requiring instead a recognisable right arm in the original position. In contrast, the ability of ScaR to bind enterococcal sequence suggests it is capable of recognising the right arm in both the original and repeat positions. These observations suggest that the presence of a right arm sequence in either the original or repeat positions may be sufficient for recognition and binding, depending on the cognate repressor protein. In other words, it may be unnecessary to have recognisable right arm sequence in both original and repeat positions. This raises the question of whether repressor proteins (and in particular those like ScaR that are capable of recognising right arm sequence in both positions) are capable of recognising right arm sequence in positions intermediate between these two. Several promoters were identified in a search of the *E. faecalis* genome (discussed later) which contain what appear to be EfaR boxes that are 15 to 17 bp long. It would be interesting to ascertain if EfaR and ScaR are indeed capable of binding such sequences.

A

	Lee *et al.* Alignment	Reassessed Alignment
tox	ATAA**TTAGGATAGCTTTACCTAA**TTAT	ATAA**TTAGGATAGCTTTA**CCTAATTAT
IRP1	ATTT**TTAGGTTAGCCAAACCTTT**GTTG	ATTT**TTAGGTTAGCCAAA**CCTTTGTTG
IRP2	CCGC**GCAGGGTAGCCTAACCTAA**ACCG	CCGC**GCAGGGTAGCCTAA**CCTAAACCG
IRP3	TCTA**TTAGGTGAGACGCACCCAT**CGGA	TCTA**TTAGGTGAGACGCA**CCCATCGGA
IRP4	TTTC**ATTACTAACGCTAACCTAA**GTAG	TTAC**TAACGCTAACCTAA**GTAG
IRP5	AGCA**CTAGGATTGCCTACACTTA**CTAA	AGCA**CTAGGATTGCCTAC**ACTTACTAA
Consensus	**TTAGGTTAGCCTAACCTAA**	TTAGGnTAGCCTAA
Identity	3555534554544556544	44656 55555545

B

Fig. 5.6. (A) Sequences in the left hand column are presented as aligned as by Lee *et al.* (Lee *et al.*, 1997). Sequences in the right hand column have been reinterpreted as containing 14 bp motifs, rather than 19 bp ones. (B) Comparison of *C. diphtheriae* DtxR binding sequences (*tox*, IRP and *hmuO*) with sequences from *E. faecalis* (*efaC*), *E. faecium* (*efmC*) and *S. epidermidis* (*sitC*).

Analysis of the *E. faecalis* V583 Genome for EfaR Boxes

As mentioned above, EfaR is predicted to bind to 14 bp DNA sequences similar to that bound by *S. epidermidis* SirR. It was hypothesised that some EfaR boxes in the *E. faecalis* genome might be identified by conducting a computer search. The 14 bp consensus described above and *E. faecalis* and *E. faecium* sequences already proven in this work to be bound by EfaR were used to search an unannotated copy of the *E. faecalis* V583 genome downloaded from TIGR. This *E. faecalis* genome was also searched using *C. diphtheriae* and *M. tuberculosis* binding sequences (for which there existed evidence of DtxR or IdeR binding) that had been reinterpreted as 14 bp motifs. Because of the lack of conservation amongst the central four bases in the 14 bp sequences (see above), the sequences were modified slightly in that any combination of nucleotides was allowed in the central four bases, e.g. the proven sequence TAAGGCAAACCTAA was modified to produce the search template TAAGGNNNNCCTAA. Two unproven sequences were also used. One was found by examination of the putative promoter of the second *E. faecalis efaCBA* homologue. The second, TCAGGNNNNCCTAA, was the result of a single fortuitous typing error (in what was intended to be the *hmuO* sequence). The search was conducted using the computer programme Vector NTI Viewer (InforMax). To identify putative EfaR-regulated genes, the BLASTX facility available at TIGR was used to investigate genomic sequence flanking candidate EfaR boxes.

Candidates located within about 200 bp of ORF start codons were considered to be promoter candidates, on the basis that repressive operators belonging to other prokaryotic regulatory systems have been documented as far as 200 bp upstream of

transcription start sites. Since transcription start sites are always upstream of the ORF start codon, this also puts any such sequences within 200 bp of the transcription initiation points. The eight search templates that successfully located EfaR boxes in putative promoters yielded a total of seventeen sequences in sixteen promoters (including the already-proven *efaC* promoter boxes) and a further sixteen sequences within ORFs (**Table 5.3A**), a ratio of approximately 1:1 (**Table 5.3B**). Even after adding six further templates that had yielded thirteen ORF-only sequences, the overall ratio of promoter- and ORF-located sequences was still only approximately 1:1.7. In contrast, assuming a random distribution of EfaR boxes in the *E. faecalis* genome, it would be expected that the number of boxes found inside ORFs should far exceed those in putative promoters. Assuming an average ORF length of 1000 bp, a random distribution should result in the discovery of five EfaR boxes in ORFs for every one found in a 200 bp promoter. Hence, the results suggest that the locations of most (if not all) of the EfaR boxes found were unlikely to have been the result of chance.

The fourteen genes preceded by putative EfaR boxes could be divided into several categories. The first group consists of genes encoding four putative manganese transporters: *efaCBA* and a fellow Psa homologue (the two homologues are henceforth designated *efaCBA1* and *efaCBA2*, respectively), and two *mntH* homologues (*mntH1* and *mntH2*). The second group of two genes is concerned with ribonucleotide synthesis or degradation. The *prsA* gene is divergently transcribed from *efaCBA* and encodes ribose-phosphate pyrophosphokinase (involved in purine and pyrimidine ribonucleotide biosynthesis). A second gene, *rpiR*, encodes a putative RpiR-type phosphosugar-binding transcriptional regulator which represses

the expression of *rpiB* (which encodes ribose-phosphate isomerase B, an enzyme involved in the metabolism of ribose). The product of the *pnp* gene, a putative polyribonucleotide nucleotidyltransferase, is involved in RNA degradation while the fourth gene of the group encodes a putative homologue of endoribonuclease L-PSP (thought to interfere in the elongation step of protein synthesis by degrading RNA (Morishita *et al.*, 1999)). The last group contains miscellaneous genes: two genes encoding ABC transporter permease (substrates thought to be oligopeptide and nitrate) genes, and various single genes encoding an alcohol dehydrogenase, a metallo-β-lactamase, a hexapeptide-repeat containing-acetyltransferase (maltose metabolism) (Boos *et al.*, 1981), a *B. subtilis* ComEC-like competence protein which plays a role in DNA uptake into the cell (Inamine and Dubnau, 1995) and a transposase.

Table 5.3A. *E. faecalis* genes possibly containing EfaR boxes within their putative promoter or ORF regions.

Locus*	EfaR box sequence	Notes
EF2074	TTAGGTGCGCCTAA	Box 1 of *efaCBA1*
EF2074	TAAGGCAAACCTAA	Box 2 of *efaCBA1*
EF2073	TTAGGCGCACCTAA	Box 1 of *efaCBA1*, 88 bp upstream of divergently transcribed *prsA*, ribose-phosphate pyrophosphokinase, purine biosynthesis
EF2073	TTAGGTTTGCCTTA	Box 2 of *efaCBA1*, 143 bp upstream of *prsA*, ribose-phosphate pyrophosphokinase
EF0575	TTAGGCTTGACTAA	*efaCBA2*, putative Mn transporter, 69 bp upstream of ORF
EF1901	TTAGGTGTGCCTAA	*mntH1*, putative Mn transporter, 64 bp upstream of ORF
EF1057	TTAGGTGTACCTAA	*mntH2*, putative Mn transporter,73 bp upstream of ORF
EF0115	TTAGGAATGCCTTA	Endoribonuclease L-PSP, RNA degradation,163 bp upstream of ORF
EF1054	TAAGGCTTTCCTAA	ABC transporter permease protein, substrate nitrate(?),119 bp upstream of ORF
EF1066	ATTGGATAACCTTA	Hexapeptide-repeat containing-acetyltransferase, 119 bp upstream of ORF
EF1826	ATTGGTGTTCCTTA	Alcohol dehydrogenase, energy generation by fermentation, 67 bp upstream of ORF
EF3107	ATTGGCAACCCTTA	ABC oligopeptide transporter permease protein, 90 bp upstream of ORF
EF2447	TGCGTACGCCCTAA	ComEC/Rec2 DNA internalisation-related competence protein, 85 bp upstream of ORF
EF2434	TTTGGGATTCCTAA	RpiR family phosphosugar-binding transcriptional regulator, 110 bp upstream of ORF
EF2359	TTTGGAAACCCTAA	Transposase, overlapping start of ORF
EF3064	TTAGGAACTACTAA	*pnp*, Polyribonucleotide nucleotidyltransferase, RNA degradation, 92 bp upstream of ORF
EF2432	TTAGGAGGTCCTGA	Metallo-β-lactamase, penicillin resistance, 2 bp upstream of ORF
In an ORF		
EF1042	TTAGGGACGCCTAA	Multidrug resistance protein
EF1559	TTAGGAACCCCTGC	Conserved hypothetical protein
EF1643	TTAGGGAAACCTGC	Conserved hypothetical protein

EF1294	TTAGGCTATCCTGC	tRNA pseudouridine synthase B
EF1737	TTAGGCGCTACTAA	Hypothetical protein
EF0471	TTAGGTTCAACTAA	Ribonucleoside-diphosphate reductase 2, alpha subunit
EF2150	TGCGTGTATCCTAA	FemAB family protein, peptidoglycan cross-linking
EF0018	ATTGGTGTTCCTTA	BglG family transcriptional antiterminator
EF1185	ATTGGCATGCCTTA	Conserved hypothetical protein
EF1732	TTAGGTATGCGTTA	ABC transporter, ATP-binding/permease protein
EF2379	TTAGGAACGCGTTA	Prolyl-tRNA synthetase gene
EF2379	TTAGGCCCAACGCA	Prolyl-tRNA synthetase gene
EF2472	TTAGGGTTGCCTTA	O-sialoglycoprotein endopeptidase, protein degradation
EF1989	TTAGGAACACCAAA	Ferrochelatase
EF0330	TTAGGAAACCCAAA	SNF2/RAD54 family helicase
EF2403	TTAGGGCAACCAAA	Membrane protein
EF2348	TTTGGTGTACCTAA	Hypothetical protein
EF2364	TTTGGTGATCCTAA	Xanthine/uracil permease family protein
EF2875	TTTGGTTCACCTAA	Acetyl-CoA carboxylase, carboxyl transferase alpha subunit
EF2455	TTTGGCAATCCTAA	Hypothetical protein
EF0888	TTTGGTACGCCTAA	Hypothetical protein
EF0646	TGAGGATGTCCTAA	NAD-dependent epimerase/dehydratase family protein
EF1362	TTAGGGAAACCTCA	Hypothetical protein
EF3174	TCAGGGAATCCTAA	Conserved hypothetical protein
EF2378	TTAGGTTAACCTGA	DNA polymerase III, alpha subunit
EF0726	TTAGGGTTACCTGA	Glutamyl-tRNA(Gln) amidotransferase, B subunit
EF1519	TTAGGGGCTCCTGA	Cation-transporting ATPase, E1-E2 family
EF2469	TTAGGTATTCCTGA	Transcriptional regulator
EF3248	TCAGGTATCCCTAA	Hypothetical protein

*TIGR locus

Table 5.3B. Comparison of incidences of EfaR boxes being found within putative promoters versus within ORFs.

Putative EfaR box	Search Template*	Number in putative promoters	Number in ORFs	Total
Sequences located in promoters or ORFs				
TTAGGNNNNCCTAA	14 bp consensus	4	1	5
TAAGGNNNNCCTAA	*efaC1* box II	2	0	2
TTAGGNNNNCCTTA	*efaC1* box II	2	1	3
TTAGGNNNNACTAA	*efaC2*	2	2	4
ATTGGNNNNCCTTA	*fxbA* R	3	2	5
TGCGTNNNNCCTAA	IRP3 R	1	1	2
TTTGGNNNNCCTAA	IRP1 R	2	5	7
TTAGGNNNNCCTGA	*hmuO*e R	1	4	5
Totals		17	16	33
Sequences located in ORFs only				
TTAGGNNNNCGTTA	IRP4 R	0	2	2
TTAGGNNNNCCTGC	IRP2 R	0	3	3
TTAGGNNNNCCAAA	IRP1	0	3	3
TTAGGNNNNACGCA	IRP3	0	1	1
TGAGGNNNNCCTAA	*hmuO*	0	1	1
TTAGGNNNNCCTCA	*hmuO* R	0	1	1
TCAGGNNNNCCTAA	*hmuO*e	0	2	2
Totals		0	13	13
Sequences absent from genome				
CTAGGNNNNCCTAC	IRP5	0	0	0
GCAGCNNNNCCTAA	IRP2	0	0	0
TAACGNNNNCCTAA	IRP4	0	0	0
TAAGGNNNNCCAAT	*fxbA*	0	0	0
GTAGGNNNNCCTAG	IRP5 R	0	0	0
TTAGTNNNNCCTAA	*efaC2* R	0	0	0

*The letter R denotes reversed and complemented sequences. *hmuOe* is the *hmuO* sequence with C instead of G at the second position.

The putative promoters of the divergently-transcribed *efaCBA* and *prsA* appear to be unique in *E. faecalis* in their possession of two easily-recognisable tandemly-located EfaR boxes. Very recently, the divergently-transcribed *M. tuberculosis bfd* and *bfrA*

genes were also shown to possess two tandem boxes (Gold *et al.*, 2001), albeit located rather further upstream (231 and 207 bp) of the ORF. IdeR represses transcription of *bfd* in the presence of iron by binding to the IdeR boxes. The *bfrA* gene possesses three transcription start sites, termed P_{low1}, P_{low2} and P_{high} after the level of expression from these sites. The P_{low} sites are repressed by IdeR and iron, with each of the IdeR boxes overlaps a –10 element corresponding to one of the P_{low} sites. P_{high} is located 110 bp upstream of the ORF. In the absence of iron, the P_{low} sites are activated whereas P_{high} is repressed, and vice-versa in the presence of iron. Deletion of the P_{low} sites removed IdeR-dependent repression during high iron conditions but also reduced transcription from P_{high}. P_{high} activity was abolished when both IdeR and the IdeR boxes were absent, implying that the presence of IdeR was required for transcription from P_{high}. Gold *et al.* suggested that IdeR repressor bound to the IdeR boxes might contact and activate RNA polymerase bound to P_{high} (Gold *et al.*, 2001). A *B. subtilis* precedent exists: knocking out the IdeR-like protein MntR suppressed the expression of an *mntA* promoter:reporter fusion instead of resulting in constitutive high-level expression of the reporter as expected (Que and Helmann, 2000). The net effect of the three *M. tuberculosis* promoters is that expression of bacterioferritin, an iron storage protein, is reduced during iron starvation and increased when the metal is abundant (Gold *et al.*, 2001), suggesting a mechanism for iron homeostasis. Is such a regulatory model universal where there are tandem DtxR-like binding sequences? Results from our Northern and Western blot analysis of *E. faecalis efaC* suggest otherwise, as expression of EfaA and transcription of *efaCBA* were completely abolished in the presence of manganese, rather than reduced. Furthermore, the EfaR boxes in the putative *efaC* promoter are very much closer to the ORF (box II is 16 bp upstream of the ORF), making the

existence of a further RNA polymerase binding site between the EfaR boxes and the ORF unlikely.

The *prsA* gene is divergently transcribed from *efaCBA* and encodes ribose-phosphate pyrophosphokinase (alternatively known as 5-phospho-D-ribosyl-α-1-pyrophosphate synthase, PRPPase). This enzyme is involved in the conversion of ribose-5-phosphate to 5-phospho-D-ribosyl-α-1-pyrophosphate, an essential precursor in the synthesis of both nucleotides and pyridine nucleotide co-enzymes. Very recently, the *B. subtilis* homologue has been shown to utilise divalent metal cations in its active site (Eriksen *et al.*, 2002). Mg^{2+} is thought to be the enzyme's preferred co-factor although other divalent cations can apparently substitute for Mg^{2+}, albeit at lower activity. Interestingly, an EfaR box was also found in the putative *rpiR* promoter. In *E. coli*, RpiR has been shown to be a transcriptional repressor of *rpiB*, which encodes ribose phosphate isomerase. This enzyme is a part of the non-oxidative branch of the pentose-phosphate pathway involved in ribose-5-phosphate catabolism (Sorensen and Hove-Jensen, 1996).

In an *E. coli fur* mutant, it was discovered that *sodB* mRNA half-life was decreased (Dubrac and Touati, 2000). Hence, it is most interesting that EfaR boxes should have been discovered in the promoters of two genes encoding proteins which degrade RNA, as this raises the possibility that EfaR has a role in post-transcriptional regulation, supporting the proposition that EfaR may have a global regulatory role. One gene encodes an endoribonuclease L-PSP (liver perchloric acid-soluble protein) homologue. Experiments conducted using a cell-free protein synthesis system derived from rabbit reticulocyte lysate demonstrated that L-PSP interferes in the elongation step of protein

synthesis by degrading mRNA (Morishita *et al.*, 1999). Expression of a human homologue was shown to be up-regulated when monocytes are induced to differentiate into macrophages (Schmiedeknecht *et al.*, 1996). The product of the *pnp* gene, polyribonucleotide nucleotidyltransferase (or polynucleotide phosphorylase, PNP), is thought to play a role in cold shock adaptation in *E. coli*. On subjection to cold shock, *E. coli* growth stops transiently in an acclimation phase, during which cold shock proteins (CSPs) are induced. Synthesis of CSPs is reduced whereas synthesis of other proteins is resumed at the end of this phase. PNP, in conjunction with CsdA RNA helicase, has been shown to suppress of CSP production by selective degradation of CSP mRNA (Yamanaka and Inouye, 2001). Why L-PSP and PNP might be part of the EfaR regulon is not immediately apparent, but it is possible that in *E. faecalis*, the two proteins plays a role in adaptation to manganese deficiency and/or oxidative stress rather than (or perhaps in addition to) cold shock.

Because of its close homology to *efaCBA*, the other Psa-like operon (referred henceforth as *efaCBA2*) had also been predicted to possess two EfaR boxes. Only one such box was found however. Unexpectedly, on further examination of the promoter sequence, a well-conserved putative Fur or PerR box was found between the EfaR box and the ORF (**Fig. 5.7**). This finding is intriguing in that it suggests an interplay between the EfaR regulon and either or both the Fur and Per regulons (discussed later).

```
TCTTCAACAACAGAAAAAATCCAAATTTAAAAATTTAGGCTTGACTAAA
                                    EfaR Box
ATATTATTTTTGTGTACCATACAATCGTGATAATCATTATCATTTAGAAG
                                  Fur Box
          M  L  E  V  K  K  L  T  V  C  Y  N  D  F
GAGAAAGTTATGTTAGAAGTGAAAAAGCTTACTGTTTGTTATAACGATTT
```

Fig. 5.7. Putative promoter of *efaC2*, with potential EfaR and Fur-type boxes displayed underlined and in bold type.

There is a precedent for the co-existence of EfaR-like and Fur-like binding sequences in a single promoter: the promoter of *E. coli mntH* was recently revealed to be repressed by the EfaR homologue MntR (in the presence of Mn^{2+}) and by Fur (in the presence of Fe^{2+}) (Patzer and Hantke, 2001). MntH is homologous to the eukaryotic NRAMP (natural resistance-associated macrophage proteins) family of divalent transition metal transporters. Prokaryotic MntH homologues have been described in both Gram-negative (e.g. *E. coli* and *Salmonella typhimurium*) and Gram-positive bacteria (e.g. *B. subtilis*). Unlike their eukaryotic cousins, which are thought to be concerned mainly with iron transport, the prokaryotic homologues are proton-dependent transporters highly selective for manganese, and to a lesser extent iron (Que and Helmann, 2000; Kehres *et al.*, 2000; Makui *et al.*, 2000). Examination of the rate of iron uptake by Gram-negative MntH homologues in one study led its authors to suggest that it was not physiologically relevant (Kehres *et al.*, 2000). Inactivation of *E. coli* and *S. typhimurium mntH* made the cells more sensitive to peroxide stress, and expression of an *mntH::lacZ* promoter:reporter fusion in *S. typhimurium* was shown to be maximal about three hours following invasion of macrophage cells (Kehres *et al.*, 2000). It is thought that bacterial MntH homologues may play a protective role against reactive

oxygen species deployed by macrophages as part of the host's defence against infection. Alternatively, or in addition, bacteria trapped in vacuoles may utilise MntH to compete against mammalian natural resistance-associated macrophage proteins (NRAMP) for Mn^{2+}. A study of the *B. subtilis mntH* did not find iron-responsive regulation (Que and Helmann, 2000), which is consistent with the lack of a readily-identifiable Fur box in the Gram-positive promoter sequence. Examination of 250 bp of sequence preceding the two *E. faecalis mntH* genes did not reveal any obvious Fur or PerR boxes.

There exists a large family of hexapeptide acetyltransferase enzymes characterised by members' possession of tandem repeats of a six-residue hexapeptide repeat sequence motif. Members of the family are involved in a variety of activities, ranging from cell wall biosynthesis, amino acid metabolism to antibiotic detoxification. The *E. faecalis* homologue identified here is most closely related to *Lactococcus lactis* subsp. *lactis* maltose O-acetyltransferase (50% identical and 70% similar). In *E. coli*, maltose O-acetyltransferase participates in the metabolism of maltose (Boos *et al.*, 1981).

The metallo-β-lactamase identified here as possessing a prospective EfaR binding sequence belongs to a class of β-lactamases which make use of one or two zinc ions to cleave β-lactam antibiotics. Such β-lactamases are not inactivated by clinically useful β-lactamases inhibitors and hence pose a particular problem clinically (Wang *et al.*, 1999).

Does EfaR really play a global regulatory role? The results so far seem promising. The computer search undertaken was conservative at best, as only a limited number of

mostly proven sequences were used to search the *E. faecalis* genome. Yet, thirteen genes in addition to *efaC* have been identified that potentially contain EfaR boxes in their promoters. Ultimately, however, defining the complete EfaR regulon will require that the promoter sequences of all genes possessing sequences even remotely-related to the consensus be screened by EMSA. Those promoters found to be shifted by EfaR should then be tested by *in vivo* expression assays to confirm the degree of negative (or even positive!) regulation, and by footprinting to determine the sequences involved. One EfaR homologue, *M. smegmatis* IdeR, had previously been shown not only to regulate total siderophore biosynthesis but was also required for effective oxidative defence (Dussurget *et al.*, 1996). Very recently, a computer-based search in *M. tuberculosis* yielded over forty different genes containing putative IdeR boxes (Gold *et al.*, 2001) of which six had been verified by experimentation (*mbtA*, *mbtB*, *mbtI*, *rv3402*, *bfd* and *bfrA*). No information was provided on how many sequences was found in ORFs. The gene products were involved in activities as diverse as iron acquisition, amino acid biosynthesis and penicillin resistance. It should be borne in mind that *M. tuberculosis* has two DtxR-like homologues, and so may be expected to possess a larger number of genes containing DtxR-like binding sequences than may necessarily be typical of most bacteria. Nevertheless, all the evidence seems to suggest a global regulatory role for DtxR-like proteins.

Operator Factors Affecting Repressibility

Variations in repressibility can be achieved by the specific nucleotide sequences of operator sites, which influence the binding affinity of repressors for the sites in question. Lee *et al.* found that IRP3 had a weaker iron response that other promoters and needed higher iron concentrations in order to be activated (Lee *et al.*, 1997). A study of the effect of nucleotide substitutions was conducted with mobility shift assays and with *in vivo* assays of transcription using promoter:reporter fusions. A T-to-C substitution (compared to consensus) at the 3' end of the binding recognition sequence was discovered to be responsible for the lack of repressibility of the sequence (Lee and Holmes, 2000). The highly repressible IRP1 has a T at that position. When this nucleotide was converted to a C, drastic reduction in repressibility was observed, supporting the IRP3 finding (Lee and Holmes, 2000).

Additionally, variations in repressibility can also be a function of the positioning of the operator relative to the promoter. The role of a repressor is to prevent transcription initiation, and classically, this is achieved by interference with the actions of RNA polymerase. Repression has been found to be more effective when the repressor binds an operator located downstream of the -10 element compared to one upstream of the -35 element, probably because the initiation region and transcription start site are occluded in the former (Lanzer and Bujard, 1988). However, it has also been found that binding to an operator located in the region between the -35 and -10 elements confers the highest degree of repression, which is attributed to simultaneous occlusion of both promoter elements by a repressor (Lanzer and Bujard, 1988). As the promoter of the *efaCBA* operon contains two operators which flank the putative -35 and -10 elements,

this might explain the completeness of repression observed during Northern analysis of *efaCBA* expression following growth in Mn^{2+}-supplemented medium compared with the less-effective repression observed with other homologues (which all possess a single operator in their respective promoters).

An explanation for the presence of EfaR boxes within ORFs, and one which could also serve to explain the tightness of repression of *efaCBA* expression, involves DNA looping. It is known that tight repression of promoters can be achieved via the use of auxiliary operators which result in stable DNA looping, which presumably leads to exclusion of RNA polymerase from its binding sites or blocks the activities of that enzyme. The *lac* promoter is a prime example (Oehler *et al.*, 1990). It has been shown that LacI is a tetramer capable of binding two operators simultaneously and that full repression requires simultaneous binding, which results in looping of the intervening DNA (Oehler *et al.*, 1990). It is entirely plausible that the tightness of repression of *efaCBA* expression is caused by stable DNA looping by EfaR bound to the two EfaR boxes in the *efaC* promoter. The EfaR boxes found to be located in the various ORFs may likewise serve as auxiliary operators for promoter-located EfaR boxes not revealed by the search templates used in this study.

Do the EfaR, Fur and Per Regulons Interact?

The observation that *efaCBA2* has both an EfaR box AND a Fur or PerR box suggests an intriguing interplay between the EfaR regulon and either or both the Fur and Per regulons. BLASTP analysis of the *E. faecalis* genome revealed that *E. faecalis*

possesses three proteins homologous to members of the Fur family of metal-responsive transcriptional regulators: one highly homologous to Fur, a second similar to PerR and a third like Zur (unpublished observations). Whether the *efaC2* sequence contains a functional Fur or PerR box is uncertain. Classically, the Fur box has been considered to be a palindrome of two inverted 9 bp repeats. The *E. faecalis* sequence could be interpreted as a well-conserved Fur box with five mismatches out of 19 bases (**Fig. 5.8**). If the Fur box is considered as an array of hexameric repeats as suggested by Escolar *et al.* (Escolar *et al.*, 1999), then the degree of conservation is even greater, with only one mismatch. It could also be a slightly less well conserved PerR box (compared with the *S. aureus* consensus as derived by Horsburgh *et al.* (Horsburgh *et al.*, 2001b)), with three mismatches out of seventeen bases. Either way, this box is unusual in that a search of the *E. faecalis* genome using the various consensus sequences failed to reveal further boxes. Examination both by eye and by ClustalW alignment of the promoters of various genes known to be regulated by PerR in *S. aureus* and *B. subtilis* also failed to reveal any sequences recognisable as resembling either the *S. aureus* PerR binding consensus or a *B. subtilis* one defined by Chen *et al.* (Chen *et al.*, 1995).

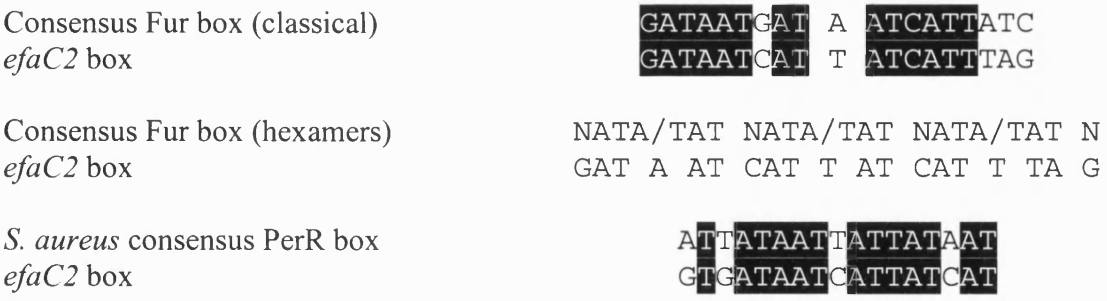

Fig. 5.8. Comparison of the *efaCBA2* putative Fur/PerR box against consensus sequences.

In *S. aureus*, Fur is an iron-dependent transcriptional repressor, whose regulon is primarily concerned with iron acquisition (Horsburgh *et al.*, 2001a). However, excessive levels of intracellular iron can result in significant oxidative stress through formation of deleterious hydroxyl radicals via the Fenton reaction. Significantly, *S. aureus* contains a Fur homologue, PerR, which is a Mn^{2+}-responsive transcriptional repressor and whose regulon comprises genes encoding proteins for oxidative stress resistance (e.g. catalase and thioredoxin reductase) and for iron storage (e.g. ferritin and the ferritin-like Dps homologue, MrgA) (Horsburgh *et al.*, 2001a). PerR also represses Fur expression (Herbig and Helmann, 2001). PerR hence exerts both direct and indirect control over iron homeostasis, the latter via regulation of Fur and the former via iron storage proteins, thus protecting *S. aureus* from the harmful effects of reactive oxygen species. Since the likely function of *efaCBA2* is Mn^{2+} transport, it would be logical to conclude that the box in its promoter is a PerR one.

A model can thus be envisaged in which EfaR modulates the activity of the PerR and Fur operons via Mn^{2+}, or rather, Mn^{2+} modulates the activity of the PerR and Fur operons via the EfaR regulon. A scarcity of manganese with which to combat oxidative stress triggers the expression of the EfaR and PerR regulons, the former to scavenge manganese and the latter to produce non-manganese-requiring enzymatic alternatives with which to mop up reactive oxygen species. Fur expression is no longer repressed by PerR, resulting in reduced intake of potentially-damaging Fe^{2+} ions. Supporting a role for the EfaR regulon in oxidative stress defence are findings that the MntH transporter of *S. enterica* ssp. *typhimurium* was induced by peroxide (Kehres *et al.*, 2000) and that deactivation of the *efaA* homologue of *S. pneumoniae*, *psaA*, resulted in hypersensitivity to oxidative stress (Tseng *et al.*, 2002). Also, as mentioned earlier in

the chapter, the enzyme G6PD is likely co-transcribed with EfaR and is indirectly linked with oxidative stress defence since G6PD-deficient *S. typhimurium* were shown to have increased susceptibility to reactive oxygen species (Lundberg *et al.*, 1999). In practice, the scheme described above is likely to prove overly-simplistic – the reality would probably be that the activity of each of the regulons is carefully titrated against each other in order to maintain an optimal balance of the two ions, i.e. oxidative defence must be balanced against the requirement for iron in a variety of essential roles.

Chapter 6: Conclusions

In this study, it has been demonstrated that EfaCBA expression is regulated by Mn^{2+}. It was shown by Western and Northern blot analyses that under Mn^{2+}-limiting conditions, expression of EfaA and transcription of *efaCBA* were derepressed. In contrast, the addition of Mn^{2+} to the growth medium repressed EfaA expression and *efaCBA* transcription. Other divalent transition metal ions such as Fe^{2+} and Zn^{2+} had no discernible effect. The sequence homology and Mn^{2+}-dependent expression of EfaCBA suggests that it is a likely Mn^{2+} permease. Analysis of the putative promoter region of *efaCBA* identified two 14 bp motifs similar to that previously found in the promoter of the *S. epidermidis* cluster 9 operon *sitCBA* which had been shown to be shifted in a metal-dependent manner by the DtxR-like *S. epidermidis* protein SirR (Hill *et al.*, 1998). A novel 222-residue *E. faecalis* protein homologous to *S. gordonii* ScaR, *C. diphtheriae* DtxR and *S. epidermidis* SirR was identified and characterised. This protein, termed EfaR, was shown via gel mobility shift and DNaseI assays to bind to the 14 bp motifs in the putative promoter region of the *efaCBA* operon in the presence of Mn^{2+}. Hence, EfaR is the likely mediator of the manganese-dependent repression of the *efaCBA* operon. It is suggested that this represents a new paradigm for metalloregulation in enterococci. This work, to the best of our knowledge, is the first to demonstrate manganese regulation in *E. faecalis*.

Computer-based analysis of the unannotated *E. faecalis* V583 genome obtained from the Institute of Genomic Research revealed the presence of putative EfaR binding

sequences in a number of genes, suggesting that EfaR may have a global regulatory role. In addition to *efaC*, at least thirteen genes were identified potentially containing EfaR boxes in their promoters. However, confirmation that each of these genes are part of the EfaR regulon will require that they be screened by gel mobility shift assay. Ideally, they would then be tested by *in vivo* expression assays to confirm the degree of regulation, and by footprinting to determine the sequences involved. Also, it would be important to map the transcriptional start points of *efaC* and those other genes. Once established as being part of the EfaR regulon, the roles of the proteins encoded by those gene could then be characterised. In particular, uptake experiments involving radioisotopes should be performed to demonstrate that EfaCBA, EfaCBA2 and the NRAMP homologues are also Mn^{2+} transporters, as this would confirm the importance of EfaR in Mn^{2+} homeostasis. Characterisation of the other gene products could reveal the extent to which EfaR influences cellular processes. Ultimately, if a global regulatory role can be established for EfaR and the EfaR regulon shown to play an important role in *E. faecalis* virulence, EfaR could prove a suitable target for the development of novel antimicrobial agents. Hence, there exists plenty of scope for future work.

In **Chapter 5**, it was proposed that the completeness of Mn^{2+}-mediated repression of *efaCBA* may have been the result of DNA looping between the two EfaR boxes leading to the exclusion of RNA polymerase. The hypothesis could be tested in future work by assessing the effect on expression of promoter:reporter fusions of a) abolishing either of the EfaR boxes, and b) varying the distance between the two operator sequences. If the involvement of a DNA looping mechanism in the

repression of *efaCBA* could be confirmed, it would lend support to the hypothesis that those putative EfaR boxes found in ORFs may play a similar role.

One of the original goals of this study had been to investigate the roles of EfaA and EfaR in manganese transport and in the pathogenicity of *E. faecalis*, and in particular in the pathogenesis of enterococcal endocarditis, via the use of isogenic mutants. Unfortunately, such investigations could not be carried out because attempts in our laboratory to construct isogenic *E. faecalis* JH2-2 *efaA* and *efaR* mutants by allelic replacement and insertion duplication have to date been unsuccessful. There have been several reports of successful insertion duplication using other strains of *E. faecalis* such as OG1RF, suggesting that those strains may be more amenable to homologous recombination than JH2-2 and hence should be tested. Another problem encountered was relatively low enterococcal transformation efficiency. A possible alternative which might avoid the problems attendant to electroporation of *E. faecalis* is conjugation. Shuttle vectors containing an origin of conjugal transfer (*ori*T) can be used to transfer DNA from *E. coli* to Gram-positive species. As this method of introducing vector DNA technique was used successfully to inactivate the *E. faecalis* autolysin gene (Teng *et al.*, 1998), it should be considered for future use.

The *E. faecalis* genome contains three Fur homologues which are homologous to *S. aureus* Fur, PerR and Zur, respectively (unpublished observations). In *S. aureus*, PerR is a Mn^{2+}-responsive regulator of an oxidative stress regulon and also mediates the repression of Fur (Horsburgh *et al.*, 2001b). Hence, the identification of a Fur- or PerR-like box alongside an EfaR box in the putative promoter region of *efaCBA2*

was most intriguing. The potential existence of a link between Fur and/or PerR and the EfaR regulon is an exciting prospect, implying as it does a complex intertwining of iron and manganese homeostasis with antioxidant defence. An interplay of these systems during pathogenesis can be envisaged. For future work, it would be fascinating to identify and confirm the genes comprising the *E. faecalis* Fur and PerR regulons in addition to that of the EfaR regulon, and to study the interactions of these regulons.

It has been shown here that EfaR was able to bind to the putative promoter of *efmCBA*, an *E. faecium* cluster 9 operon, in the presence of Mn^{2+}. Transcription of this operon has also been found to be Mn^{2+}-responsive but repression was not as complete as seen with *efaCBA* (unpublished observation). Examination of the nucleotide sequence of the putative *efmC* promoter fragment revealed only one binding sequence, which may explain the difference in degree of repression observed. Work in this species has to date been hampered by the lack of readily-available sequence information and even greater problems with lack of transformability (compared with *E. faecalis*). Considering the clinical importance of *E. faecium* strains (particularly with regards to vancomycin resistance), however, further investigation into metal-regulation in *E. faecium* is warranted. Differences between *E. faecalis* and *E. faecium* might be revealed that could be exploited in the development of agents selective for either species. An example of such a selective agent already exists in the form of linezolid, which is bacteriostatic for *E. faecium* but not *E. faecalis*.

To summarise, the findings suggest that EfaCBA is a Mn^{2+} permease. The results also identify EfaR as a novel *E. faecalis* metal-dependent DNA-binding protein, and the likely regulator of EfaCBA expression. Examination of the *E. faecalis* chromosome suggests that EfaR in conjunction with Mn^{2+} may have a global regulatory role, with at least thirteen genes containing possible EfaR boxes in their promoters. These include both *E. faecalis* genes encoding putative NRAMP proteins, homologues of which have been shown in other bacteria to be Mn^{2+} transporters. EfaR may also interact directly or indirectly with regulons controlled by *E. faecalis* Fur and PerR homologues. Further investigation of these metalloregulatory systems will improve our understanding of this bacterium and may yield novel targets for new antimicrobial agents which may help to overcome clinical problems with resistance to currently available antibiotics.

Chapter 7: References

Short Protocols in Molecular Biology (1999) Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). New York: John Wiley & Sons.

Aitchison, E.J., Lambert, P.A., Smith, E.G., and Farrell, I.D. (1987) Serodiagnosis of *Streptococcus faecalis* endocarditis by immunoblotting of surface protein antigens. *J. Clin. Microbiol.* **25**: 211-215.

Alloing, G., Granadel, C., Morrison, D.A., and Claverys, J.-P. (1996) Competence pheromone, oligopeptide permease, and induction of competence in *Streptococcus pneumoniae. Mol. Microbiol.* **21**: 471-478.

Althaus, E.W., Outten, C.E., Olson, K.E., Cao, H., and O'Halloran, T.V. (1999) The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. *Biochemistry* **38**: 6559-6569.

An, F.Y., Clewell, D.B. (2002) Identification of the cAD1 sex pheromone precursor in *Enterococcus faecalis. J. Bacteriol.* **184**: 1880-1887.

Andersen, R.N., Ganeshkumar, N., and Kolenbrander, P.E. (1993) Cloning of the *Streptococcus gordonii* PK488 gene, encoding an adhesin which mediates coaggregation with *Actinomyces naeslundii* PK606. *Infect. Immun.* **61**: 981-987.

Antonov, P.A., Maximova, V.A., and Pancheva, R.P. (1993) Heat shock and osmotically dependent steps by DNA uptake after *Escherichia coli* electroporation. *Biochim. Biophys. Acta* **1216**: 286-288.

Archibald, F.S. (1983) *Lactobacillus plantarum*, an organism not requiring iron. *FEMS Microbiol. Lett.* **19**: 29-32.

Archibald, F.S. (1986) Manganese: its acquisition by and function in the lactic acid bacteria. *Crit. Rev. Microbiol.* **13**: 63-109.

Bagg, A., Neilands, J.B. (1987b) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**: 5471-5477.

Bagg, A., Neilands, J.B. (1987a) Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* **51**: 509-518.

Barford, D. (1996) Molecular mechanisms of the protein serine threonine phosphatases. *Trends Biochem. Sci.* **21**: 407-412.

Bartsevich, V.V., Pakrasi, H.B. (1995) Molecular identification of an ABC transporter complex for manganese - analysis of a cyanobacterial mutant strain impaired in the photosynthetic oxygen evolution process. *EMBO J.* **14**: 1845-1853.

Bartsevich, V.V., Pakrasi, H.B. (1996) Manganese transport in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **271**: 26057-26061.

Bashkirov, V.I., Khasanov, F.K., and Prozorov, A.A. (1987) Illegitimate recombination in *Bacillus subtilis*: nucleotide sequences at recombinant DNA junctions. *Mol. Gen. Genet.* **210**: 578-580.

Bearden, S.W., Perry, R.D. (1999) The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. *Mol. Microbiol.* **32**: 403-414.

Berlin, J.A., Abrutyn, E., Strom, B.L., Kinman, J.L., Levison, M.E., Korzeniowski, O.M., Feldman, R.S., and Kaye, D. (1995) Incidence of infective endocarditis in the Delaware Valley, 1988-1990. *Am. J. Cardiol.* **76**: 933-936.

Berry, A.M., Paton, J.C. (1996) Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **64**: 5255-5262.

Bhavnani, S.M., Ballow, C.H. (2000) New agents for Gram-positive bacteria. *Curr. Opin. Microbiol.* **3**: 528-534.

Biswas, I., Gruss, A., Ehrlich, S.D., and Maguin, E. (1993) High-efficiency gene inactivation and replacement system for Gram-positive bacteria. *J. Bacteriol.* **175**: 3628-3635.

Boland, C.A., Meijer, W.G. (2000) The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi. FEMS Microbiol. Lett.* **191**: 1-5.

Bonow, R.O., Carabello, B., de Leon, A.C., Edmunds, L.H., Fedderly, B.J., Freed, M.D., Gaasch, W.H., McKay, C.R., Nishimura, R.A., O'Gara, P.T., O'Rourke, R.A., Rahimtoola, S.H., Ritchie, J.L., Cheitlin, M.D., Eagle, K.A., Gardner, T.J., Garson, A., Gibbons, R.J., Russell, R.O., Ryan, T.J., and Smith, S.C. (1998) Guidelines for the management of patients with valvular heart disease. Executive summary. A report of the American College of Cardiology American Heart Association task force on practice guidelines (committee on management of patients with valvular heart disease). *Circulation* **98**: 1949-1984.

Boos, W., Ferenci, T., and Shuman, H.A. (1981) Formation and excretion of acetylmaltose after accumulation of maltose in *Escherichia coli. J. Bacteriol.* **146**: 725-732.

Boyd, J., Oza, M.N., and Murphy, J.R. (1990) Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae. Proc. Natl. Acad. Sci. USA* **87**: 5968-5972.

Bsat, N., Chen, L., and Helmann, J.D. (1996) Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. *J. Bacteriol.* **178**: 6579-6586.

Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P., and Helmann, J.D. (1998) *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol. Microbiol.* **29**: 189-198.

Burnette-Curley, D., Wells, V., Viscount, H., Munro, C.L., Fenno, J.C., Fives-Taylor, P., and Macrina, F.L. (1995) FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. *Infect. Immun.* **63**: 4669-4674.

Caron, F., Gold, H.S., Wennersten, C.B., Farris, M.G., Moellering, R.C., and Eliopoulos, G.M. (1997) Influence of erythromycin resistance, inoculum growth phase, and incubation time on assessment of the bactericidal activity of RP 59500 (quinupristin-dalfopristin) against vancomycin-resistant *Enterococcus faecium*. *Antimicrob. Agents Chemother.* **41**: 2749-2753.

Cetinkaya, Y., Falk, P., and Mayhall, C.G. (2000) Vancomycin-resistant enterococci. *Clin. Microbiol. Rev.* **13**: 686-707.

Chander, M., Setlow, B., and Setlow, P. (1998) The enzymatic activity of phosphoglycerate mutase from Gram-positive endospore-forming bacteria requires Mn^{2+} and is pH sensitive. *Can. J. Microbiol.* **44**: 759-767.

Chen, L., Keramati, L., and Helmann, J.D. (1995) Coordinate regulation of *Bacillus subtilis* peroxide stress gene by hydrogen peroxide and metal ions. *Proc. Natl. Acad. Sci. USA* **92**: 8190-8194.

Chen, Y.W., Dekker, E.E., and Somerville, R.L. (1995) Functional analysis of *E. coli* threonine dehydrogenase by means of mutant isolation and characterisation. *Biochim. Biophys. Acta* **1253**: 208-214.

Cheton, P.L.B., Archibald, F.S. (1988) Manganese complexes and the generation and scavenging of hydroxyl free radicals. *Free Radic. Biol. Med.* **5**: 325-333.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., and Zervos, M.J. (1993) Plasmid-associated haemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob. Agents Chemother.* **37**: 2474-2477.

Christianson, D.W. (1997) Structural chemistry and biology of manganese metalloenzymes. *Progress In Biophysics & Molecular Biology* **67**: 217.

Church, G.M., Gilbert, W. (1984) Genomic Sequencing. *Proc. Natl. Acad. Sci. USA* **81**: 1991-1995.

Claverys, J.P. (2001) A new family of high-affinity ABC manganese and zinc permeases. *Res. Microbiol.* **152**: 231-243.

Clewell, D.B. (1990) Movable genetic elements and antibiotic resistance in enterococci. *Eur. J. Microbiol. Infect. Dis.* **9**: 90-102.

Clewell, D.B., An, F.Y., White, B.A., and Gawron-Burke, M.C. (1985) *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn*918*). *J. Bacteriol.* **162**: 1212-1220.

Cockayne, A., Hill, P.J., Powell, N.B.L., Bishop, K., Sims, C., and Williams, P. (1998) Molecular cloning of a 32-kilodalton lipoprotein component of a novel iron-regulated *Staphylococcus epidermidis* ABC transporter. *Infect. Immun.* **66**: 3767-3774.

Coque, T.M., Patterson, J.E., Steckelberg, J.M., and Murray, B.E. (1995) Incidence of haemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from faeces of hospitalised and community-based persons. *J. Infect. Dis.* **171**: 1223-1229.

Correia, F.F., DiRienzo, J.M., McKay, T.L., and Rosan, B. (1996) *scbA* from *Streptococcus crista* CC5A: an atypical member of the *lraI* gene family. *Infect. Immun.* **64**: 2114-2121.

Coy, M., Neilands, J.B. (1991) Structural dynamics and functional domains of the Fur protein. *Biochemistry* **30**: 8201-8210.

Crosa, J.H. (1997) Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol. Mol. Biol. Rev.* **61**: 319-&.

Cruz-Rodz, A.L., Gilmore, M.S. (1990) High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. *Mol. Gen. Genet* **224**: 152-154.

de Peredo, A.G., Saint-Pierre, C., Latour, J.M., Michaud-Soret, I., and Forest, E. (2001) Conformational changes of the ferric uptake regulation protein upon metal activation and DNA binding; first evidence of structural homologies with the diphtheria toxin repressor. *J. Mol. Biol.* **310**: 83-91.

Dempsey, L.A., Dubnau, D.A. (1989) Identification of plasmid and *Bacillus subtilis* chromosomal recombination sites used for pE194 integration. *J. Bacteriol.* **171**: 2856-2865.

Diekema, D.J., Jones, R.N. (2001) Oxazolidinone antibiotics. *Lancet* **358**: 1975-1982.

Ding, X., Zeng, H., Schiering, N., Ringe, D., and Murphy, J.R. (1996) Identification of the primary metal ion-activation sites of the diphtheria *tox* repressor by X-ray crystallography and site-directed mutational analysis. *Nat. Struct. Biol.* **3**: 382-387.

Dintilhac, A., Alloing, G., Granadel, C., and Claverys, J.-P. (1997b) Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol. Microbiol.* **25**: 727-739.

Dintilhac, A., Claverys, J.-P. (1997a) The *adc* locus, which affects competence for genetic transformation in *Streptococcus pneumoniae*, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. *Res. Microbiol.* **148**: 119-131.

Dubrac, S., Touati, D. (2000) Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. *J. Bacteriol.* **182**: 3802-3808.

Dunny, G.M., Lee, L.N., and LeBlanc, D.J. (1991) Improved electroporation and cloning vector system for Gram-positive bacteria. *Appl. Environ. Microbiol.* **57**: 1194-1201.

Dunny, G.M., Leonard, B.A.B. (1997) Cell-cell communication in Gram-positive bacteria. *Annu. Rev. Microbiol.* **51**: 527-564.

Dussurget, O., Rodriguez, M., and Smith, I. (1996) An *ideR* mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. *Mol. Microbiol.* **22**: 535-544.

Eaton, T.J., Gasson, M.J. (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* **67**: 1628-1635.

Eriksen, T.A., Kadziola, A., and Larsen, S. (2002) Binding of cations in *Bacillus subtilis* phosphoribosyldiphosphate synthetase and their role in catalysis. *Protein Science* **11**: 271-279.

Escolar, L., Perez-Martin, J., and de Lorenzo, V. (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. *J. Bacteriol.* **181**: 6223-6229.

Escolar, L., Perez-Martin, J., and de Lorenzo, V. (2000) Evidence of an unusually long operator for the Fur repressor in the aerobactin promoter of *Escherichia coli*. *J. Biol. Chem.* **275**: 24709-24714.

Felmingham, D., Wilson, A.P.R., Quintana, A.I., and Gruneberg, R.N. (1992) *Enterococcus* species in urinary tract infection. *Clin. Infect. Dis.* **15**: 295-301.

Fenno, J.C., Shaikh, A., and Fives-Taylor, P. (1993) Characterisation of allelic replacement in *Streptococcus parasanguis*: transformation and homologous recombination in a 'nontransformable' streptococcus. *Gene* **130**: 81-90.

Fenno, J.C., Shaikh, A., Spatafora, G., and Fives-Taylor, P. (1995) The *fimA* locus of *Streptococcus parasanguis* encodes an ATP-binding membrane transport system. *Mol. Microbiol.* **15**: 849-863.

Fernandez-Guerrero, M.L., Verdejo, C., Azofra, J., and de Gorgolas, M. (1995) Hospital-acquired infective endocarditis not associated with cardiac surgery: an emerging problem. *Clin. Infect. Dis.* **20**: 16-23.

Flatman, J.C. (1999) Characterisation of the *Enterococcus faecalis efaA* gene product in infective endocarditis. Ph.D. Thesis. University of Bath.

Fowler, V.G., Durack, D.T. (1994) Infective endocarditis. *Curr. Opin. Cardiol.* **9**: 389-400.

Franz, C.M.A.P., Muscholl-Silberhorn, A.B., Yousif, N.M.K., Vancanneyt, M., Swings, J., and Holzapfel, W.H. (2001) Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl. Environ. Microbiol.* **67**: 4385-4389.

Friesenegger, A., Fiedler, S., Devriese, L.A., and Wirth, R. (1991) Genetic transformation of various species of *Enterococcus* by electroporation. *FEMS Microbiol. Lett.* **79**: 323-327.

Frontera, J.A., Gradon, J.D. (2000) Right-side endocarditis in injection drug users: review of proposed mechanisms of pathogenesis. *Clin. Infect. Dis.* **30**: 374-379.

Gaballa, A., Helmann, J.D. (1998) Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. *J. Bacteriol.* **180**: 5815-5821.

Gerberding, J., Gaynes, R., Horan, T., Abshire, J., Alonso-Echanove, J., Edwards, J., Emori, G., Fridkin, S., Hageman, J., Henderson, T., Killen, L., Lawton, R., McLay, C., Peavy, G., Richards, C., and Tolson, J. (1999) National

Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1990-May 1999, issued June 1999. *Am. J. Infect. Control* **27**: 520-532.

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., and Clewell, D.B. (1994) Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J. Bacteriol.* **176**: 7335-7344.

Gold, B., Rodriguez, G.M., Marras, S.A.E., Pentecost, M., and Smith, I. (2001) The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. *Mol. Microbiol.* **42**: 851-865.

Goranson-Siekierke, J., Pohl, E., Hol, W.G.J., and Holmes, R.K. (1999) Anion-coordinating residues at binding site 1 are essential for the biological activity of the diphtheria toxin repressor. *Infect. Immun.* **67**: 1806-1811.

Griffith, K.L., Wolf, R.E. (2001) Systematic mutagenesis of the DNA binding sites for SoxS in the *Escherichia coli zwf* and *fpr* promoters: identifying nucleotides required for DNA binding and transcription activation. *Mol. Microbiol.* **40**: 1141-1154.

Guzman, C.A., Pruzzo, C., and Calegari, L. (1991) *Enterococcus faecalis*: specific and non-specific interactions with human polymorphonuclear leukocytes. *FEMS Microbiol. Lett.* **84**: 157-162.

Guzman, C.A., Pruzzo, C., LiPira, G., and Calegari, L. (1989) Role of adherence in pathogenesis of *Enterococcus faecalis* urinary tract infection and endocarditis. *Infect. Immun.* **57**: 1834-1838.

Hall, H.K., Foster, J.W. (1996) The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. *J. Bacteriol.* **178**: 5683-5691.

Hantke, K. (1987) Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12: *fur* not only affects iron metabolism. *Mol. Gen. Genet.* **210**: 135-139.

Hao, Z.Q., Chen, S.L., and Wilson, D.B. (1999) Cloning, expression, and characterisation of cadmium and manganese uptake genes from *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **65**: 4746-4752.

Hardie, J.M., Whiley, R.A. (1997) Classification and overview of the genera *Streptococcus* and *Enterococcus*. *J. Appl. Microbiol.* **83**: S1-S11.

Herbig, A.F., Helmann, J.D. (2001) Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol. Microbiol.* **41**: 849-859.

Higgins, C.F. (1992) ABC Transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**: 67-113.

Hill, P.J., Cockayne, A., Landers, P., Morrissey, J.A., Sims, C.M., and Williams, P. (1998) SirR, a novel iron-dependent repressor in *Staphylococcus epidermidis*. *Infect. Immun.* **66**: 4123-4129.

Hinds, J., Mahenthiralingam, E., Kempsell, K.E., Duncan, K., Stokes, R.W., Parish, T., and Stoker, N.G. (1999) Enhanced gene replacement in mycobacteria. *Microbiology* **145**: 519-527.

Hirt, H., Erlandsen, S.L., and Dunny, G.M. (2000) Heterologous inducible expression of *Enterococcus faecalis* pCF10 aggregation substance Asc10 in *Lactococcus lactis* and *Streptococcus gordonii* contributes to cell hydrophobicity and adhesion to fibrin. *J. Bacteriol.* **182**: 2299-2306.

Hirt, H., Schlievert, P.M., and Dunny, G.M. (2002) *In vivo* induction of virulence and antibiotic resistance transfer in *Enterococcus faecalis* mediated by the sex pheromone-sensing system of pCF10. *Infect. Immun.* **70**: 716-723.

Hogevik, H., Olaison, L., Andersson, R., Lindberg, J., and Alestig, K. (1995) Epidemiologic aspects of infective endocarditis in an urban population: a 5-year prospective study. *Medicine* **74**: 324-339.

Holmes, R.K. (2000) Biology and molecular epidemiology of diphtheria toxin and the *tox* gene. *J. Infect. Dis.* **181**: S156-S167.

Horsburgh, M.J., Clements, M.O., Crossley, H., Ingham, E., and Foster, S.J. (2001b) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect. Immun.* **69**: 3744-3754.

Horsburgh, M.J., Ingham, E., and Foster, S.J. (2001a) In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J. Bacteriol.* **183**: 468-475.

Huycke, M.M., Abrams, V., and Moore, D.R. (2002) *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* **23**: 529-536.

Huycke, M.M., Gilmore, M.S. (1997) *In vivo* survival of *Enterococcus faecalis* is enhanced by extracellular superoxide production. *Adv. Exp. Med. Biol.* **418**: 781-784.

Huycke, M.M., Joyce, W., and Wack, M.F. (1996) Augmented production of extracellular superoxide by blood isolates of *Enterococcus faecalis*. *J. Infect. Dis.* **173**: 743-746.

Huycke, M.M., Moore, D., Joyce, W., Wise, P., Shepard, L., Kotake, Y., and Gilmore, M.S. (2001) Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. *Mol. Microbiol.* **42**: 729-740.

Huycke, M.M., Sahm, D.F., and Gilmore, M.S. (1998) Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerg. Infect. Dis.* **4**: 239-249.

Huycke, M.M., Spiegel, C.A., and Gilmore, M.S. (1991) Bacteraemia caused by haemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **35**: 1626-1634.

Ike, Y., Hashimoto, H., and Clewell, D.B. (1984) Haemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. *Infect. Immun.* **45**: 528-530.

Ike, Y., Hashimoto, H., and Clewell, D.B. (1987) High incidence of haemolysin production by *Enterococcus faecalis* strains associated with human parenteral infections. *J. Clin. Microbiol.* **25**: 1524-1528.

Ikeda, H. (1986) Illegitimate recombination mediated by T4 DNA topoisomerase in vitro. Recombinants between phage and plasmid DNA molecules. *Mol. Gen. Genet.* **202**: 518-520.

Ikeda, H., Aoki, K., and Naito, A. (1982) Illegitimate recombination mediated *in vitro* by DNA gyrase of *Escherichia coli*. Structure of recombinant DNA molecules. *Proc. Natl. Acad. Sci. USA* **79**: 3724-3728.

Ikeda, H., Kawasaki, I., and Gellert, M. (1984) Mechanisms of illegitimate recombination: common sites for recombination and cleavage mediated by *E. coli* DNA gyrase. *Mol. Gen. Genet.* **196**: 546-549.

Inamine, G.S., Dubnau, D. (1995) ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. *J. Bacteriol.* **177**: 3045-3051.

Inaoka, T., Matsumura, Y., and Tsuchido, T. (1999) SodA and manganese are essential for resistance to oxidative stress in growing and sporulating cells of *Bacillus subtilis*. *J. Bacteriol.* **181**: 1939-1943.

Isenmann, R., Schwarz, M., Rozdzinski, E., Marre, R., and Beger, H.G. (2000) Aggregation substance promotes colonic mucosal invasion of *Enterococcus faecalis* in an *ex vivo* model. *J. Surg. Res.* **89**: 132-138.

Jack, R.W., Tagg, J.R., and Raze, D. (1995) Bacteriocins of Gram-positive bacteria. *Microbiol. Rev.* **59**: 171-200.

Jacob, A.E., Hobbs, S.J. (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. *J. Bacteriol.* **117**: 360-372.

Jakubovics, N.S., Jenkinson, H.F. (2001) Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. *Microbiology* **147**: 1709-1718.

Jakubovics, N.S., Smith, A.W., and Jenkinson, H.F. (2000) Expression of the virulence-related Sca (Mn^{2+}) permease in *Streptococcus gordonii* is regulated by a diphtheria toxin metallorepressor-like protein ScaR. *Mol. Microbiol.* **38**: 140-153.

Janulczyk, R., Pallon, J., and Bjorck, L. (1999) Identification and characterisation of a *Streptococcus pyogenes* ABC transporter with multiple specificity for metal cations. *Mol. Microbiol.* **34**: 596-606.

Jenkinson, H.F. (1992) Adherence, coaggregation, and hydrophobicity of *Streptococcus gordonii* associated with expression of cell surface lipoproteins. *Infect. Immun.* **60**: 1225-1228.

Jenkinson, H.F. (1994) Cell surface protein receptors in oral streptococci. *FEMS Microbiol. Lett.* **121**: 133-140.

Jett, B.D., Jensen, H.G., Nordquist, R.E., and Gilmore, M.S. (1992) Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infect. Immun.* **60**: 2445-2452.

Johnson, C.M. (1993) Adherence events in the pathogenesis of infective endocarditis. *Infect. Dis. Clin. North Am.* **7**: 21-36.

Kaye, D. (1985a) Changing pattern of infective endocarditis. *Am. J. Med.* **78**: 157-162.

Kaye, D. (1985b) Infective endocarditis. An overview. *Am. J. Med.* **78**: 107-109.

Kehres, D.G., Zaharik, M.L., Finlay, B.B., and Maguire, M.E. (2000) The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. *Mol. Microbiol.* **36**: 1085-1100.

Khasanov, F.K., Zvingila, D.J., Zainullin, A.A., Prozorov, A.A., and Bashkirov, V.I. (1992) Homologous recombination between plasmid and chromosomal DNA in *Bacillus subtilis* requires approximately 70 bp of homology. *Mol. Gen. Genet.* **234**: 494-497.

Kitten, T., Munro, C.L., Michalek, S.M., and Macrina, F.L. (2000) Genetic characterisation of a *Streptococcus mutans* LraI family operon and role in virulence. *Infect. Immun.* **68**: 4441-4451.

Kogoma, T. (1997) Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. *Microbiol. Mol. Biol. Rev.* **61**: 212.

Kolenbrander, P.E., Andersen, R.N., Baker, R.A., and Jenkinson, H.F. (1998) The adhesion-associated *sca* operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn^{2+} uptake. *J. Bacteriol.* **180**: 290-295.

Kolenbrander, P.E., Andersen, R.N., and Ganeshkumar, N. (1994) Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. *Infect. Immun.* **62**: 4469-4480.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D., and Rehrauer, W.M. (1994) Biochemistry of homologous recombination in *Escherichia coli* . *Microbiol. Rev.* **58**: 401-465.

Krachler, M., Rossipal, E., and Micetic-Turk, D. (1999) Concentrations of trace elements in sera of newborns, young infants, and adults. *Biol. Trace Elem. Res.* **68**: 121-135.

Kreft, B., Marre, R., Schramm, U., and Wirth, R. (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infect. Immun.* **60**: 25-30.

Kuhn, N.J., Setlow, B., Setlow, P., Cammack, R., and Williams, R. (1995) Cooperative manganese (II) activation of 3-phosphoglycerate mutase of *Bacillus megaterium*: a biological pH-sensing mechanism in bacterial spore formation and germination. *Arch. Biochem. Biophys.* **320**: 35-42.

Kusano, K., Sakagami, K., Yokochi, T., Naito, T., Tokinaga, Y., Ueda, E., and Kobayashi, I. (1997) A new type of illegitimate recombination is dependent on restriction and homologous interaction. *J. Bacteriol.* **179**: 5380-5390.

Kyte, J., Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**: 105-132.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lambert, P.A., Shorrock, P.J., Aitchison, E.J., Domingue, P.A.G., Power, M.E., and Costerton, J.W. (1990) Effect of *in vivo* growth conditions upon expression of surface protein antigens in *Enterococcus faecalis*. *FEMS Microbiol. Immunol.* **64**: 51-54.

Landman, D., Quale, J.M. (1997) Management of infections due to resistant enterococci: a review of therapeutic options. *J. Antimicrob. Chemother.* **40**: 161-170.

Lanzer, M., Bujard, H. (1988) Promoters largely determine the efficiency of repressor action. *Proc. Natl. Acad. Sci. USA* **85**: 8973-8977.

Lawrence, M.C., Pilling, P.A., Epa, V.C., Berry, A.M., Ogunniyi, A.D., and Paton, J.C. (1998) The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. *Structure* **6**: 1553-1561.

Le Cam, E., Frechon, D., Barray, M., Fourcade, A., and Delain, E. (1994) Observation of binding and polymerisation of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. *Proc. Natl. Acad. Sci. USA* **91**: 11816-11820.

Leclercq, R., Dutka-Malen, S., Brisson-Noel, A., Molinas, C., Derlot, E., Arthur, M., Duval, J., and Courvalin, P. (1992) Resistance of enterococci to aminoglycosides and glycopeptides. *Clin. Infect. Dis.* **15**: 495-501.

Lee, J.H., Holmes, R.K. (2000) Characterisation of specific nucleotide substitutions in DtxR-specific operators of *Corynebacterium diphtheriae* that dramatically affect DtxR binding, operator function, and promoter strength. *J. Bacteriol.* **182**: 432-438.

Lee, J.H., Wang, T., Ault, K., Liu, J., Schmitt, M.P., and Holmes, R.K. (1997) Identification and characterisation of three new promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun.* **65**: 4273-4280.

Lee, Y.H., Deka, R.K., Norgard, M.V., Radolf, J.D., and Hasemann, C.A. (1999) *Treponema pallidum* TroA is a periplasmic zinc-binding protein with a helical backbone. *Nat. Struct. Biol.* **6**: 628-633.

Lowe, A.M., Lambert, P.A., and Smith, A.W. (1995) Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. *Infect. Immun.* **63**: 703-706.

Lukes, A.S., Durack, D.T. (1993) Infective endocarditis. *Curr. Opin. Cardiol.* **8**: 502-510.

Lundberg, B.E., Wolf, R.E., Jr., Dinauer, M.C., Xu, Y., and Fang, F.C. (1999) Glucose 6-phosphate dehydrogenase is required for *Salmonella typhimurium* virulence and resistance to reactive oxygen and nitrogen intermediates. *Infect. Immun.* **67**: 436-438.

Makui, H., Roig, E., Cole, S.T., Helmann, J.D., Gros, P., and Cellier, M.F.M. (2000) Identification of the *Escherichia coli* K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter. *Mol. Microbiol.* **35** : 1065-1078.

Martin, M.E., Strachan, R.C., and Aranha, H. (1984) Oxygen toxicity in *Streptococcus mutans*: manganese, iron, and superoxide dismutase. *J. Bacteriol.* **159**: 745-749.

McCormick, J.K., Hirt, H., Waters, C.M., Tripp, T.J., Dunny, G.M., and Schlievert, P.M. (2001) Antibodies to a surface-exposed, N-terminal domain of aggregation substance are not protective in the rabbit model of *Enterococcus faecalis* infective endocarditis. *Infect. Immun.* **69**: 3305-3314.

McFadden, J. (1996) Recombination in mycobacteria. *Mol. Microbiol.* **21**: 205-211.

McNeil, S.A., Clark, N.M., Chandrasekar, P.H., and Kauffman, C.A. (2000) Successful treatment of vancomycin-resistant *Enterococcus faecium* bacteraemia with linezolid after failure of treatment with Synercid (quinupristin/dalfopristin). *Clin. Infect. Dis.* **30**: 403-404.

Megran, D.W. (1992) Enterococcal endocarditis. *Clin. Infect. Dis.* **15**: 63-71.

Meima, R., Haan, G.J., Venema, G., Bron, S., and de Jong, S. (1998) Sequence specificity of illegitimate plasmid recombination in *Bacillus subtilis*: possible recognition sites for DNA topoisomerase I. *Nucleic Acids Res.* **26**: 2366-2373.

Meima, R., Venema, G., and Bron, S. (1996) A positive selection vector for the analysis of structural plasmid instability in *Bacillus subtilis*. *Plasmid* **35**: 14-30.

Miller, J.F. (1994) Bacterial transformation by electroporation. *Methods Enzymol.* **235**: 375-385.

Moellering, R.C., Jr. (1995) *Enterococcus* species, *Streptococcus bovis*, and *Leuconostoc* species. In *Principles and Practice of Infectious Diseases*. Mandell, G.L., Bennett, J.E., and Dolin, R. (eds). New York: Churchill Livingstone, pp. 1826-1835.

Moellering, R.C., Jr. (1998) Vancomycin-resistant enterococci. *Clin. Infect. Dis.* **26** : 1196-1199.

Morishita, R., Kawagoshi, A., Sawasaki, T., Madin, K., Ogasawara, T., Oka, T., and Endo, Y. (1999) Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis. *J. Biol. Chem.* **274**: 20688-20692.

Morona, J.K., Morona, R., Miller, D.C., and Paton, J.C. (2002) *Streptococcus pneumoniae* capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase. *J. Bacteriol.* **184**: 577-583.

Morrison, D., Woodford, N., and Cookson, B. (1997) Enterococci as emerging pathogens of humans. *J. Appl. Microbiol.* **83**: 89S-99S.

Murray, B.E. (1990) The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* **3**: 46-65.

Murray, B.E. (1998) Diversity among multidrug-resistant enterococci. *Emerg. Infect. Dis.* **4**: 37-47.

Murray, B.E. (2000) Vancomycin-resistant enterococcal infections. *N. Engl. J. Med.* **342**: 710-721.

Muscholl-Silberhorn, A., Samberger, E., and Wirth, R. (1997) Why does *Staphylococcus aureus* secrete an *Enterococcus faecalis*-specific pheromone? *FEMS Microbiol. Lett.* **157**: 261-266.

Mylonakis, E., Calderwood, S.B. (2001) Medical progress: infective endocarditis in adults. *N. Engl. J. Med.* **345**: 1318-1330.

Nallapareddy, S.R., Qin, X., Weinstock, G.M., Hook, M., and Murray, B.E. (2000b) *Enterococcus faecalis* adhesin, *ace*, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect. Immun.* **68**: 5218-5224.

Nallapareddy, S.R., Singh, K.V., Duh, R.W., Weinstock, G.M., and Murray, B.E. (2000a) Diversity of *ace*, a gene encoding a microbial surface component recognising adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of *ace* during human infections. *Infect. Immun.* **68**: 5210-5217.

Niven, D.F., Ekins, A., and Al-Samaurai, A.A.W. (1999) Effects of iron and manganese availability on growth and production of superoxide dismutase by *Streptococcus suis*. *Can. J. Microbiol.* **45**: 1027-1032.

Noble, W.C., Virani, Z., and Cree, R.G.A. (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus* . *FEMS Microbiol. Lett.* **93**: 195-198.

Novak, R., Braun, J.S., Charpentier, E., and Tuomanen, E. (1998) Penicillin tolerance genes of *Streptococcus pneumoniae*: the ABC-type manganese permease complex Psa. *Mol. Microbiol.* **29**: 1285-1296.

Oehler, S., Eismann, E.R., Kramer, H., and Muller-Hill, B. (1990) The three operators of the *lac* operon cooperate in repression. *EMBO J.* **9**: 973-979.

Oguiza, J.A., Tao, X., Marcos, A.T., Martin, J.F., and Murphy, J.R. (1995) Molecular cloning, DNA sequence analysis, and characterisation of the *Corynebacterium diphtheriae dtxR* homologue from *Brevibacterium lactofermentum*. *J. Bacteriol.* **177**: 465-467.

Oguiza, J.A., Marcos, A.T., Malumbres, M., and Martin, J.F. (1996) The *galE* gene encoding the UDP-galactose 4-epimerase of *Brevibacterium lactofermentum* is coupled transcriptionally to the *dmdR* gene. *Gene* **177**: 103-107.

Oligino, L., Fives-Taylor, P. (1993) Overexpression and purification of a fimbria-associated adhesin of *Streptococcus parasanguis*. *Infect. Immun.* **61**: 1016-1022.

Olmsted, S.B., Dunny, G.M., Erlandsen, S.L., and Wells, C.L. (1994) A plasmid-encoded surface protein on *Enterococcus faecalis* augments its internalisation by cultured intestinal epithelial cells. *J. Infect. Dis.* **170**: 1549-1556.

Patzer, S.I., Hantke, K. (1998) The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol. Microbiol.* **28**: 1199-1210.

Patzer, S.I., Hantke, K. (2000) The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli* . *J. Biol. Chem.* **275**: 24321-24332.

Patzer, S.I., Hantke, K. (2001) Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. *J. Bacteriol.* **183**: 4806-4813.

Podbielski, A., Leonard, B.A.B. (1998) The group A streptococcal dipeptide permease (Dpp) is involved in the uptake of essential amino acids and affects the expression of cysteine protease. *Mol. Microbiol.* **28**: 1323-1334.

Podbielski, A., Pohl, B., Woischnik, M., Korner, C., Schmidt, K.H., Rozdzinski, E., and Leonard, B.A.B. (1996) Molecular characterisation of group A streptococcal (GAS) oligopeptide permease (Opp) and its effect on cysteine protease production. *Mol. Microbiol.* **21**: 1087-1099.

Podbielski, A., Spellerberg, B., Woischnik, M., Pohl, B., and Lutticken, R. (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**: 137-147.

Pohl, E., Holmes, R.K., and Hol, W.G.J. (1999b) Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain. *J. Mol. Biol.* **292**: 653-667.

Pohl, E., Holmes, R.K., and Hol, W.G.J. (1999a) Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding sites fully occupied. *J. Mol. Biol.* **285**: 1145-1156.

Posey, J.E., Gherardini, F.C. (2000) Lack of a role for iron in the Lyme disease pathogen. *Science* **288**: 1651-1653.

Posey, J.E., Hardham, J.M., Norris, S.J., and Gherardini, F.C. (1999) Characterisation of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum. Proc. Natl. Acad. Sci. USA* **96**: 10887-10892.

Pozzi, G., Guild, W.R. (1985) Modes of integration of heterologous plasmid DNA into the chromosome of *Streptococcus pneumoniae. J. Bacteriol.* **161**: 909-912.

Qin, X., Singh, K.V., Weinstock, G.M., and Murray, B.E. (2000) Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. *Infect. Immun.* **68**: 2579-2586.

Qiu, X.Y., Pohl, E., Holmes, R.K., and Hol, W.G.J. (1996) High-resolution structure of the diphtheria toxin repressor complexed with cobalt and manganese reveals an SH3-like third domain and suggests a possible role of phosphate as co-corepressor. *Biochemistry* **35**: 12292-12302.

Qiu, X.Y., Verlinde, C.L.M.J., Zhang, S.P., Schmitt, M.P., Holmes, R.K., and Hol, W.G.J. (1995) Three-dimensional structure of the diphtheria toxin repressor in complex with divalent cation co-repressors. *Structure* **3**: 87-100.

Que, Q., Helmann, J.D. (2000) Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. *Mol. Microbiol.* **35**: 1454-1468.

Quiocho, F.A., Ledvina, P.S. (1996) Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes. *Mol. Microbiol.* **20**: 17-25.

Rakita, R.M., Vanek, N.N., Jacques-Palaz, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., van Winkle, W.B., and Simon, S.I. (1999) *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect. Immun.* **67**: 6067-6075.

Rende-Fournier, R., Leclercq, R., Galimand, M., Duval, J., and Courvalin, P. (1993) Identification of the *satA* gene encoding a streptogramin A acetyltransferase in *Enterococcus faecium* BM4145. *Antimicrob. Agents Chemother.* **37**: 2119-2125.

Rich, R.L., Kreikemeyer, B., Owens, R.T., LaBrenz, S., Narayana, S.V.L., Weinstock, G.M., Murray, B.E., and Hook, M. (1999) Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis. J. Biol. Chem.* **274**: 26939-26945.

Rozdzinski, E., Marre, R., Susa, M., Wirth, R., and Muscholl-Silberhorn, A. (2001) Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilised extracellular matrix proteins. *Microb. Pathogen.* **30**: 211-220.

Russell, H., Tharpe, J.A., Wells, D.E., White, E.H., and Johnson, J.E. (1990) Monoclonal antibody recognising a species-specific protein from *Streptococcus pneumoniae. J. Clin. Microbiol.* **28**: 2191-2195.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbour, N.Y.: Cold Spring Harbour Laboratory Press.

Schaberg, D.R., Culver, D.H., and Gaynes, R.P. (1991) Major trends in the microbial aetiology of nosocomial infection. *Am. J. Med.* **91**: 72S-75S.

Schiering, N., Tao, X., Zeng, H.Y., Murphy, J.R., Petsko, G.A., and Ringe, D. (1995) Structures of the apo- and the metal ion-activated forms of the diphtheria *tox*

repressor from *Corynebacterium diphtheriae*. *Proc. Natl. Acad. Sci. USA* **92**: 9843-9850.

Schleifer, K.H., Kilpper-Balz, R. (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int. J. Syst. Bacteriol.* **34**: 31-34.

Schlievert, P.M., Gahr, P.J., Assimacopoulos, A.P., Dinges, M.M., Stoehr, J.A., Harmala, J.W., Hirt, H., and Dunny, G.M. (1998) Aggregation and binding substances enhance pathogenicity in rabbit models of *Enterococcus faecalis* endocarditis. *Infect. Immun.* **66**: 218-223.

Schmiedeknecht, G., Kerkhoff, C., Orso, E., Stohr, J., Aslanidis, C., Nagy, G.M., Knuechel, R., and Schmitz, G. (1996) Isolation and characterisation of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is upregulated upon cellular differentiation. *Eur. J. Biochem.* **242**: 339-351.

Schmitt, M.P. (1999) Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of haem and haemoglobin. *J. Bacteriol.* **181**: 5330-5340.

Schmitt, M.P., Predich, M., Doukhan, L., Smith, I., and Holmes, R.K. (1995) Characterisation of an iron-dependent regulatory protein (IdeR) of *Mycobacterium tuberculosis* as a functional homologue of the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae*. *Infect. Immun.* **63**: 4284-4289.

Schmitt, M.P., Twiddy, E.M., and Holmes, R.K. (1992) Purification and characterisation of the diphtheria toxin repressor. *Proc. Natl. Acad. Sci. USA* **89**: 7576-7580.

Schneider, E., Hunke, S. (1998) ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolysing subunits/domains. *FEMS Microbiol. Rev.* **22**: 1-20.

Shankar, V., Baghdayan, A.S., Huycke, M.M., Lindahl, G., and Gilmore, M.S. (1999) Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect. Immun.* **67**: 193-200.

Shimizu, H., Yamaguchi, H., Ashizawa, Y., Kohno, Y., Asami, M., Kato, J., and Ikeda, H. (1997) Short-homology-independent illegitimate recombination in *Escherichia coli*: distinct mechanism from short-homology-dependent illegitimate recombination. *J. Mol. Biol.* **266**: 297-305.

Shorrock, P.J., Lambert, P.A., Aitchison, E.J., Smith, E.G., Farrell, I.D., and Gutschik, E. (1990) Serological response in *Enterococcus faecalis* endocarditis determined by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **28**: 195-200.

Simmons, N.A., Ball, A.P., Eykyn, S.J., Littler, W.A., McGowan, D.A., Oakley, C.M., and Shanson, D.C. (1998) Antibiotic treatment of streptococcal, enterococcal, and staphylococcal endocarditis. *Heart* **79**: 207-210.

Singh, K.V., Coque, T.M., Weinstock, G.M., and Murray, B.E. (1998b) *In vivo* testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologues for species identification. *FEMS Immunol. Med. Microbiol.* **21**: 323-331.

Singh, K.V., Qin, X., Weinstock, G.M., and Murray, B.E. (1998a) Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. *J. Infect. Dis.* **178**: 1416-1420.

Sirard, J.C., Mock, M., and Fouet, A. (1995) Molecular tools for the study of transcriptional regulation in *Bacillus anthracis*. *Res. Microbiol.* **146**: 729-737.

Skjold, S.A., Quie, P.G., Fries, L.A., Barnham, M., and Cleary, P.P. (1987) DNA fingerprinting of *Streptococcus zooepidemicus* (Lancefield Group C) as an aid to epidemiological study. *J. Infect. Dis.* **155**: 1145-1150.

Sorensen, K.I., Hove-Jensen, B. (1996) Ribose catabolism of *Escherichia coli*: characterisation of the *rpiB* gene encoding ribose phosphate isomerase B and of the

rpiR gene, which is involved in regulation of *rpiB* expression. *J. Bacteriol.* **178**: 1003-1011.

Spatafora, G., Moore, M. (1998) Growth of *Streptococcus mutans* in an iron-limiting medium. *Methods Cell Sci.* **20**: 217-221.

Spatafora, G., Moore, M., Landgren, S., Stonehouse, E., and Michalek, S. (2001) Expression of *Streptococcus mutans fimA* is iron-responsive and regulated by a DtxR homologue. *Microbiology* **147**: 1599-1610.

Spellerberg, B., Rozdzinski, E., Martin, S., Weber-Heynemann, J., Schnitzler, N., Lutticken, R., and Podbielski, A. (1999) Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. *Infect. Immun.* **67**: 871-878.

Stojiljkovic, I., Baumler, A.J., and Hantke, K. (1994) Fur regulon in Gram-negative bacteria. *J. Mol. Biol.* **240**: 271-271.

Strom, B.L., Abrutyn, E., Berlin, J.A., Kinman, J.L., Feldman, R.S., Stolley, P.D., Levison, M.E., Korzeniowski, O.M., and Kaye, D. (2000) Risk factors for infective endocarditis - oral hygiene and non-dental exposures. *Circulation* **102**: 2842-2848.

Submuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., and Rozdzinski, E. (2000) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect. Immun.* **68**: 4900-4906.

Suhy, D.A., O'Halloran, T.V. (1996) Metal-responsive gene-regulation and the zinc metalloregulatory model. *Metal Ions in Biological Systems* **32**: 557-578.

Sun, L., vanderSpek, J., and Murphy, J.R. (1998) Isolation and characterisation of iron-independent positive dominant mutants of the diphtheria toxin repressor DtxR. *Proc. Natl. Acad. Sci. USA* **95**: 14985-14990.

Sutcliffe, I.C., Russell, R.R.B. (1995) Lipoproteins of Gram-positive bacteria. *J. Bacteriol.* **177**: 1123-1128.

Sutcliffe, I.C., Tao, L., Ferretti, J.J., and Russell, R.R.B. (1993) MsmE, a lipoprotein involved in sugar transport in *Streptococcus mutans*. *J. Bacteriol.* **175**: 1853-1855.

Swartz, M.N. (1994) Hospital-acquired infections: diseases with increasingly limited therapies. *Proc. Natl. Acad. Sci. USA* **91**: 2420-2427.

Tam, R., Saier, M.H., Jr. (1993) Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* **57** : 320-346.

Tanimoto, K., An, F.Y., and Clewell, D.B. (1993) Characterisation of the *traC* determinant of the *Enterococcus faecalis* haemolysin-bacteriocin plasmid pAD1: binding of sex pheromone. *J. Bacteriol.* **175**: 5260-5264.

Tao, L., LeBlanc, D.J., and Ferretti, J.J. (1992) Novel streptococcal-integration shuttle vectors for gene cloning and inactivation. *Gene* **120**: 105-110.

Tao, X., Boyd, J., and Murphy, J.R. (1992) Specific binding of the diphtheria *tox* regulatory element DtxR to the *tox* operator requires divalent heavy-metal ions and a 9-base-pair interrupted palindromic sequence. *Proc. Natl. Acad. Sci. USA* **89**: 5897-5901.

Tao, X., Murphy, J.R. (1993) Cysteine-102 is positioned in the metal-binding activation site of the *Corynebacterium diphtheriae* regulatory element DtxR. *Proc. Natl. Acad. Sci. USA* **90**: 8524-8528.

Tao, X., Schiering, N., Zeng, H.Y., and Murphy, J.R. (1994) Iron, DtxR, and the regulation of diphtheria toxin expression. *Mol. Microbiol.* **14**: 191-197.

Tao, X., Zeng, H.Y., and Murphy, J.R. (1995) Transition metal ion activation of DNA binding by the diphtheria *tox* repressor requires the formation of stable homodimers. *Proc. Natl. Acad. Sci. USA* **92**: 6803-6807.

Tardat, B., Touati, D. (1993) Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. *Mol. Microbiol.* **9**: 53-63.

Teng, F., Murray, B.E., and Weinstock, G.M. (1998) Conjugal transfer of plasmid DNA from *Escherichia coli* to enterococci: a method to make insertion mutations. *Plasmid* **39**: 182-186.

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penades, J.R., and Lasa, I. (2001) The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl. Environ. Microbiol.* **67**: 4538-4545.

Tseng, H.J., McEwan, A.G., Paton, J.C., and Jennings, M.P. (2002) Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. *Infect. Immun.* **70**: 1635-1639.

Twigg, P.D., Parthasarathy, G., Guerrero, L., Logan, T.M., and Caspar, D.L.D. (2001) Disordered to ordered folding in the regulation of diphtheria toxin repressor activity. *Proc. Natl. Acad. Sci. USA* **98**: 11259-11264.

Uttley, A.H.C., Collins, C.H., Naidoo, J., and George, R.C. (1988) Vancomycin-resistant enterococci. *Lancet* **1**: 57-58.

Vanek, N.N., Simon, S.I., Jacques-Palaz, K., Mariscalco, M.M., Dunny, G.M., and Rakita, R.M. (1999) *Enterococcus faecalis* aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor Type 3-mediated mechanism. *FEMS Immunol. Med. Microbiol.* **26**: 49-60.

Vasil, M.L., Ochsner, U.A. (1999) The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol. Microbiol.* **34**: 399-413.

Viscount, H.B., Munro, C.L., Burnette-Curley, D., Peterson, D.L., and Macrina, F.L. (1997) Immunisation with FimA protects against *Streptococcus parasanguis* endocarditis in rats. *Infect. Immun.* **65**: 994-1002.

Wang, G., Wylie, G.P., Twigg, P.D., Caspar, D.L.D., Murphy, J.R., and Logan, T.M. (1999) Solution structure and peptide binding studies of the C-terminal Src homology 3-like domain of the diphtheria toxin repressor protein. *Proc. Natl. Acad. Sci. USA* **96**: 6119-6124.

Wang, Z.G., Fast, W., Valentine, A.M., and Benkovic, S.J. (1999) Metallo-β-lactamase: structure and mechanism. *Current Opinion In Chemical Biology* **3**: 614-622.

Wang, Z.O., Schmitt, M.P., and Holmes, R.K. (1994) Characterisation of mutations that inactivate the diphtheriae toxin repressor gene (*dtxR*). *Infect. Immun.* **62**: 1600-1608.

Watanakunakorn, C., Burkert, T. (1993) Infective endocarditis at a large community teaching hospital, 1980-1990: a review of 210 episodes. *Medicine* **72**: 90-102.

Weinberg, E.D. (1997) The *Lactobacillus* anomaly: total iron abstinence. *Perspect. Biol. Med.* **40**: 578-583.

Wells, C.L., Moore, E.A., Hoag, J.A., Hirt, H., Dunny, G.M., and Erlandsen, S.L. (2000) Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalisation by cultured enterocytes. *Infect. Immun.* **68**: 7190-7194.

Westenberg, D.J., Guerinot, M.L. (1997) Regulation of bacterial gene expression by metals. *Adv. Genet.* **36**: 187-238.

Westerlund, B., Korhonen, T.K. (1993) Bacterial proteins binding to the mammalian extracellular matrix. *Mol. Microbiol.* **9**: 687-694.

White, A., Ding, X.C., vanderSpek, J.C., Murphy, J.R., and Ringe, D. (1998) Structure of the metal-ion-activated diphtheria toxin repressor/*tox* operator complex. *Nature* **394**: 502-506.

Williams, A.M., Rodrigues, U.M., and Collins, M.D. (1991) Intrageneric relationships of enterococci as determined by reverse transcriptase sequencing of small-subunit rRNA. *Res. Microbiol.* **142**: 67-74.

Wirth, R. (1994) The sex pheromone system of *Enterococcus faecalis*. More than just a plasmid-collection mechanism? *Eur. J. Biochem.* **222**: 235-246.

Working Party Report of the British Society for Antimicrobial Chemotherapy (1985) Antibiotic treatment of streptococcal and staphylococcal endocarditis. Report of a working party of the British Society for Antimicrobial Chemotherapy. *Lancet* **2**: 815-817.

Yamanaka, K., Inouye, M. (2001) Selective mRNA degradation by polynucleotide phosphorylase in cold shock adaptation in *Escherichia coli*. *J. Bacteriol.* **183**: 2808-2816.